MORPHOLOGY, MOLECULES AND MATING BEHAVIOR: AN INTEGRATIVE STUDY OF POPULATION DIVERGENCE AND SPECIATION IN WIDESPREAD SEPSID FLIES (SEPSIDAE: DIPTERA)

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SUMMARY

The work presented in this dissertation explores processes of selection and speciation acting on diverging populations in two widespread sepsid species (Sepsidae: Diptera). The main focus was on investigating sexual selection, sexual size dimorphism (SSD) and incipient speciation in the nearctic and palaearctic species *Sepsis punctum* (Fabricius 1794) (Chapters 1-4). In addition, divergence in reproductive behavior and morphology was also addressed in the neotropical species *Archisepsis diversiformis* (Ozerov 1993) (Chapter 5).

In **chapter one**, a unique cross-continental reversal in SSD is comprehensively examined in 4 European (EU) and 3 North American (NA) populations of the dung fly *S. punctum*. The differential equilibrium model explains SSD as the evolutionary outcome of consistent differences in natural and sexual selection between the sexes. Using a combined approach including common garden experiments and fitness component assessment in the laboratory, this chapter explicitly tested the equilibrium model of SSD. First it was established that SSD was male-biased in EU and female-biased in NA. The intensity of sexual selection increased with male body size and operational sex ratio in the EU populations and was significantly stronger than in NA populations. Fecundity selection on female body size increased strongly for egg number and weakly for egg volume, however equally on both continents. Finally, viability selection on body size in terms of intrinsic (physiological) adult lifespan in the laboratory was overall nil and did not vary significantly across all seven populations. Although it is impossible to prove causality, these results confirmed the differential equilibrium model of SSD whereby differences in sexual selection intensity account for the reversal in SSD in EU vs. NA populations.

Chapter two investigates the relationship between pre- and post-copulatory investment in *S. punctum* using an extensive comparative study of mating behavior and internal reproductive morphology. Theory predicts that males have a limited amount of resources to invest in reproduction, suggesting a trade-off between traits that enhance mate acquisition and those enhancing fertilization success. The geographic reversal in SSD between the continents (reported in Chapter 1) was found to be accompanied by differential investment in pre- versus post-copulatory traits. EU populations exhibited higher re-mating rates with larger males acquiring more matings; males have consequently evolved relatively larger testes exhibiting steeper hyper-allometry with

body size. NA populations, in sharp contrast, displayed much reduced, if any, effect of body size on those traits. Instead, NA males showed an increased investment in mate acquisition prior to copulation, with more mounting attempts and a distinctive abdominal courtship display that was completely absent in EU. Interestingly, there was also a west-east gradient in the intensity of the display in NA. Relative female spermathecal size was similar on both continents, and there was no direct evidence for the co-evolution of male and female internal reproductive morphology. By comparing allopatric populations of the same species that apparently have evolved different mating systems and consequently SSD (Chapter 1), this study indirectly demonstrates differential investment in pre- vs. post-copulatory mechanisms increasing reproductive success.

Chapter three represents a phylogeographic analysis using both a maternally inherted mitochondrial marker (cytochrome oxidase subunit I - *COI* gene fragment) and six autosomal microsatellite markers to address the underlying genetic structure among twelve *S. punctum* populations (7 EU; 5 NA). Allopatric populations of this widespread species exhibited clear genetic differentiation between and even within continents. The *COI* gene fragment yielded high haplotype diversity, and maximum parsimony and haplotype network analysis consistently recovered three geographic clusters: (i) Northern and Central EU, (ii) Southern EU and (iii) NA. The neutrality tests of Tajima's D and Fu's F_S revealed some negative values but there was no clear evidence of past demographic expansion in these groups. Additionally, admixture analysis of the microsatellite data recovered eight distinct geographic clusters that followed a spatial differentiation in genetic structure within the continents exhibiting clear isolation-by-distance. Thus, the spatial patterns of genetic variation support the shift in mating system and associated changes in behavior and SSD (Chapters 1 & 2) indicative of incipient speciation in this species.

Chapter four represents the first exploratory study of volatile organic compounds (VOCs; including cuticular hydrocarbons) in three species *Themira biloba, Nemopoda nitidula* and *S. punctum* (both from EU and NA) using gas chromatography with mass selective detection. VOCs play an important role in insect chemical communication and recent research on dipteran species suggests that VOCs can mediate reproductive isolation among closely related species and populations. This study identified 29 compounds, of which 22 have been previously reported as pheromones involved in aggregation, sex or alarm signals in various insects. The three species differed in VOC profiles and interestingly, nine putative *punctum*-specific compounds were identified that could be potentially associated with the male osmerteria. These glandular substance-producing organs are only found on the male hind tibiae of sepsid flies and are involved

in copulatory behavior. Three fatty acids and one saturated hydrocarbon, undecane, were only detected in the European populations, a result of either drift or divergent selection. Future studies including behavioral assays are needed to detail the significance of these compounds in the sexual selection context.

Finally, **chapter five** presents a detailed integrative study of mating behavior and sexual morphology in Costa Rica (CR) and Panama (PAN) populations of the widespread neotropical sepsid fly *A. diversiformis.* Comparative work across different taxa suggests that rapid diversification in reproductive traits is pivotal for the evolution of species diversity, and that behavioral cues in particular can evolve much faster than many other types of traits. This study documents that despite strong overall similarities in courtship repertoires, (i) some behavioral elements performed during mating were clearly population-specific, and (ii) that both populations exhibited a degree of pre-mating isolation when tested one-on-one. Nevertheless, population crosses produced viable F1 offspring after extended exposure to hetero-population males in group mating trials. Additionally, (iii) morphometric analysis indicated that the populations differed significantly in wing shape but only moderately in male fore femur shape and not at all in male genital shape. Finally, (iv) a comparison of the fast-evolving *COI* gene fragment showed that individuals from Costa Rica & Panama were genetically highly similar, forming a strong monophyletic cluster with uncorrected pair-wise distances only ranging from 0.5-1.6% between the two populations. Thus, the study implies that the behavioral differences between the populations have arisen rather rapidly, suggesting that both directional and stabilizing selection were operating strongly on reproductive isolating mechanisms at early stages of diversification in this neotropical fly.

The research presented here reiterates the importance of extensive within-species studies particularly among diverging populations using multiple methods. It also demonstrates the need for integrative work, including detailed behavioral, morphological and molecular data in investigating the effects of selection and speciation among widespread species.

ZUSAMMENFASSUNG

Die vorliegende Dissertation untersucht Mechanismen der Selektion und der Artbildung bei divergierenden Populationen zweier weit verbreiteter Schwingfliegenarten (Sepsidae: Diptera). Im Vorgrund standen Untersuchungen der Rolle der natürlichen und sexuellen Selektion für die Artbildung sowie den Körpergrössendimorphismus zwischen den Geschlechtern bei der weit verbreiteten Art *Sepsis punctum* (Fabricius 1794) (Kapitel 1-4). Zusätzlich wurden Populationsunterschiede in Paarungsverhalten und Morphologie auch an *Archisepsis diversiformis* (Ozerov 1993) untersucht (Kapitel 5).

Kapital eins ist eine Untersuchung von 4 europäischen und 3 nordamerikanischen Populationen von *S. punctum*, die sich kontinental im Paarungsverhalten und Geschlechtsdimorphismus unterscheiden, eine in dieser Ausprägung bislang einzigartige Situation. Nordamerikanische Weibchen sind grösser als Männchen, während dies bei europäischen Populationen umgekehrt ist. Theoretisch resultiert bei einer gegebenen Tierart ein geschlechtlicher Körpergrössendimorphismus (KGD), wenn die Vor- und Nachteile der Grösse bei Männchen und Weibchen zu einem unterschiedlichen evolutionären Gleichgewicht führen. Experimentelle Laboruntersuchungen zeigten, dass die sexuelle Selektion auf die Männchengrösse in Europa viel stärker ist als in Nordamerika, und sie ist auch insgesamt stärker, wenn mehr Männchen um die Weibchen konkurrierten. Grössere Weibchen hatten ebenfalls einen Selektionsvorteil, da sie mehr Nachkommen produzieren können, doch dieser Vorteil war in Europa und Nordamerika in etwa gleich. Gegenselektion in Form höherer Mortalität grösserer Tiere konnte nicht festgestellt werden. Insgesamt bestätigen diese Befunde das Gleichgewichtsmodell der Evolution des KGD bei *S. punctum*.

Im **Kapitel zwei** wurde der Zusammenhang zwischen prä- und post-kopulatorischen Reproduktionsmechanismen bei 9 kontinentalen Populationen von *S. punctum* untersucht. Männchen haben für die Reproduktion nur begrenzte Ressourcen zur Verfügung. Deshalb erwartet man eine Abwägung (engl. *trade-off*) zwischen der Investition in Merkmale, die den Zugang zu Paarungspartnern erhöhen und solchen, die den Befruchtungserfolg erhöhen. Die im Kapitel eins gefundene kontinentale Umkehrung des Grössendimorphismus geht tatsächlich einher mit unterschiedlichen Investitionen in prä- vs. post-kopulatorische Eigenschaften. Europäische Fliegen paaren sich häufiger, und die Männchen haben folglich vergleichsweise grössere Hoden, was einem höheren Aufwand in post-kopulatorische Spermienproduktion gleichkommt. Die weiblichen

Spermienlagerungsorgane sind im Verhältnis jedoch auf beiden Kontinenten ungefähr gleich gross, was nicht für ihre Koevolution mit der männlichen Hodengrösse spricht. Dagegen umwerben nordamerikanische Männchen ihre Weibchen mit einem energieaufwändigen prä-kopulatorischen Balztanz, der in Europa gänzlich fehlt. Dieser Balztanz zeigt zudem (genetische) Intensitätsunterschiede zwischen westlichen und östlichen Populationen in Nordamerika. Europäische Männchen bespringen stattdessen relativ wahllos die Weibchen, klammern sich mit unterschiedlichem Erfolg an diesen fest und versuchen, sie zu begatten, werden von den Weibchen aber oft vor der Kopulation wieder abgeschüttelt. Durch diesen Vergleich diversifizierter kontinentaler Populationen einer Art konnte unsere Studie indirekt den theoretisch erwarteten *trade-off* zwischen prä- und post-koplatorischen Investitionen belegen.

Kapitel drei ist eine phylogeographische Analyse der genetischen Differenzierung von kontinentalen *S. punctum*-Populationen unter Verwendung von sechs nuklearen Mikrosatelliten-Markern sowie einem mitochondrialen Gen (COI-Fragment). Europäische und nordamerikanische Populationen dieser weit verbreiteten Art zeigen deutliche genetische Unterschiede, und auch innerhalb der Kontinente zeigt sich eine gewisse Differenzierung. Verschiedene Analysen der unterschiedlichen Marker ergaben stets drei geographische Gruppen: (i) Nord- und Mitteleuropa, (ii) Südeuropa, und (iii) Nordamerika. Außerdem unterschied eine sog. *Admixture*-Analyse der Mikrosatelliten acht verschiedene Gruppen mit einer klaren räumlichen genetischen Struktur innerhalb der Kontinente sowie *Isolation-by-Distance*. So unterstützt unsere populationsgenetische Untersuchung die in Morphologie und Verhalten (Kapitel eins & zwei) gefundenen Unterschiede und weist auf beginnende Artbildung hin.

Flüchtige organische Verbindungen (fOV) spielen eine wichtige Rolle bei der chemischen Kommunikation von Insekten, und Forschungen an verschiedenen Dipterengruppen legen nahe, dass fOV reproduktive Isolation zwischen nah verwandten Arten oder Populationen bedingen kann. In **Kapital vier** wurde in einer ersten Studie die Rolle der fOV bei der Paarung von *S. punctum* sowie zwei weiteren Sepsidenarten (*Themira biloba, Nemopoda nitidula*) mittels Gaschromatographie und Massenspektroskopie untersucht. Es wurden 29 Verbindungen identifiziert, von denen 22 bereits bei anderen Insekten in verschiedensten Kontexten (Paarung, Aggregation, Alarmsignale) beschrieben wurden. Die Gesamtprofile der drei untersuchten Arten waren unterschiedlich, und interessanterweise wurden 9 für *S. punctum* spezifische Verbindungen identifiziert, die möglicherweise mit der bei vielen Sepsiden vorhandenen männlichen Hinterbeindrüse (Osmeterium) assoziiert sind und bei der Paarung eine Rolle spielen. Vier Komponenten wurden exklusiv nur bei europäischen *S. punctum* festgestellt. Weitere Studien,

insbesondere Verhaltenstests, sind vonnöten, um die Funktion dieser diversen organischen Verbindungen bei der Reproduktion aufzuschlüsseln.

Kapitel fünf präsentiert eine detaillierte vergleichende Studie des Paarungsverhaltens und der Morphologie zweier Populationen der weit verbreiteten amerikanischen Sepsidenart *Archisepsis diversiformis* aus Costa Rica und Panama. Studien an verschiedensten Arten deuten an, dass schnelle Diversifizierung von Reproduktionsorganen, und insbesondere des Reproduktionsverhaltens, die Evolution der Artenvielfalt vorantreiben. Unsere Studie belegt, dass (i) einige während der Paarung gezeigte Verhaltensweisen eindeutig populationsspezifisch sind. (ii) Beide Populationen zeigen einen gewissen Grad der Trennung, da gemischte Paarungen selten vorkamen. Trotzdem produzierten Populationskreuzungen lebensfähige Nachkommen. (iii) Morphometrische Analysen zeigten ausserdem, dass sich die Populationen deutlich in ihrer Flügelform, jedoch nur mäßig im männlichen Vorderbein und überhaupt nicht in den männlichen Genitalstrukturen unterschieden. (iv) Letztlich belegte ein Vergleich des COI-Genfragments, dass beide Populationen genetisch sehr ähnlich sind. In ihrer Gesamtheit impliziert die Studie, dass die Unterschiede zwischen den Populationen im Verhalten und der Flügelform schneller evoluieren als Unterschiede in der Morphologie primärer oder sekundärer sexueller Organe, was nicht der gängigen Auffassung entspricht.

Die hier präsentierten Forschungsarbeiten belegen die Notwendigkeit von vergleichenden Untersuchungen unterschiedlicher Populationen einer gegebenen Art, um die Artbildung auf mikroevolutionärem Niveau zu dokumentieren und besser zu verstehen. Dabei ist es von Vorteil, wenn diverse verhaltensbiologische, morphologische, evolutionsbiologische und molekulare Methoden integriert werden.

GENERAL INTRODUCTION

Understanding the role of ecological processes and evolutionary forces in driving phenotypic divergence among lineages resulting in speciation is a paramount pursuit in evolutionary biology. Geographical separation, local adaptation and divergent selection often facilitate diversification, through the accumulation and maintenance of genetic variation between species (Coyne and Orr 2004). Reproductive traits such as conspicuous secondary morphological ornaments, elaborate courtship behavior, or even overall body size can be important for both interspecific mate recognition as well as intraspecific mate preference (Dobzhansky and Mayr 1944). Although much of the work on reproductive isolation involves already evolved species, it is particularly interesting to study diverging populations of widespread species that are in intermediate stages of diversification, evolution in action, so to speak (Gröning and Hochkirch 2008; Wojcieszek and Simmons 2012). Ongoing processes of incipient speciation require that these groups of individuals acquire means of isolation so as to restrict the gene flow between them, which can occur during the pre-mating, post-mating/pre-zygotic, or post-zygotic phases of sexual interactions (Seehausen et al. 1997; Panhuis et al. 2001; Coyne and Orr 2004). Sexual selection can be particularly instrumental in generating discriminating mechanisms among populations as well as morphological and/or behavioral barriers to reproduction (Fairbairn and Preziosi 1996; Emerson and Ward 1998; Kraushaar and Blanckenhorn 2002; Rugman-Jones and Eady 2008).

Sexual selection

Sexual selection, as originally conceived by Darwin, describes the variation in reproductive success due to differences among individual males in acquiring mates (Darwin 1871). Extensive research over the last decades in a broad range of taxa has revealed an extraordinary diversity of morphological, physiological and behavioral adaptations that serve to enhance a male's own fertilization success relative to that of other males (Andersson 1994; Arnqvist and Rowe 2005; Rowe et al. 2006). Variation in such mating signals and associated preferences in mate choice between groups of individuals can result in sexual isolation (Zeh and Zeh 2007). These differences could arise between populations as a consequence of ecological separation or genetic drift, but more often than not, the degree to which reproductive isolation is maintained and reinforced is determined through sex-specific selection acting on pre- and post-mating traits (Coyne and Orr 2004; Cox and Calsbeek 2010).

Sexual size dimorphism

Species and even populations within a species can differ greatly in body size between the sexes. Sexual selection on body size has attracted considerable theoretical and empirical interest as it influences patterns of sexual size dimorphism (SSD) and causes responses in morphology, behavior and other related traits (Andersen 1994; Fairbairn 1997; Blanckenhorn 2005; Drovetski et al. 2006; Blanckenhorn et al. 2007; Fairbairn et al. 2007; Serrano-Meneses et al. 2007; Teuschl et al. 2007). Optimal size associated with the maximum fitness often varies for males and females, and according to the differential equilibrium model of the evolution of SSD, dimorphism in body size arises when the net effects of sexual and natural selection differ between the sexes (Andersson 1994; Preziosi and Fairbairn 2000; Blanckenhorn 2005). For instance, male-biased SSD is primarily attributed to increased reproductive success of larger males, whereas female-biased SSD is usually associated with strong fecundity selection in terms of increased offspring production of larger females (Blanckenhorn et al. 2007; Stillwell et al. 2010). Fecundity and sexual selection for larger individuals is presumably held in check by viability selection, counteracting forces favoring small size in terms of survival (Blanckenhorn 2000). Genetic, developmental and phylogenetic constraints additionally play a role in defining body size differences between the sexes (Badyaev 2002; Lindenfors et al. 2002; Ramos et al. 2005).

Pre- versus post-copulatory sexual selection

It is clear that sexual selection often extends far beyond the initiation of copulation. Post-copulatory processes such as cryptic female choice (Eberhard 1985, 1996) and sperm competition (Parker 1970; Simmons 2001, 2005), as well as sexual conflict over control of fertilization (Parker 1979; Arnqvist and Rowe 2005; Parker 2006), are recognized as important determinants of reproductive success in polyandrous species that mate multiply. Males have limited resources to invest in reproduction, which they must allocate to mate acquisition as well as successful inseminations and fertilizations, suggesting a fundamental trade-off between traits that enhance mating success and those that influence fertilization success, the combination of which is expected to vary with the mating system (Simmons and Emlen 2006; Parker and Pizzari 2010). This is reciprocally linked to female re-mating behavior, whereby higher rates could intensify sperm competition and post-copulatory sexual selection but may relax male competition over access to females. The opposite is expected if females rarely re-mate, implying relaxed sperm competition but perhaps more intense pre-copulatory selection and competition among males prior to mating (Reuter et al. 2008).

Divergence in morphology, mating behavior and molecular data

Sexually dimorphic structures and genitalia are often highly diverse, with numerous studies providing evidence that sexual selection contributes strongly to variation in such morphological traits (Eberhard 1985; Arnqvist and Danielsson 1999; Takami and Sota 2007). These characters evolve rapidly and can be important for diagnosing species because recently diverged taxa often differ only with respect to these structures (Ramos et al. 2005; Ingram et al. 2008; Puniamoorthy et al. 2008). Non-morphological characters, namely those involved in mating behavior also play an important role in generating sexual isolation (Simmons et al. 2001; Vedenina et al. 2007; Luan et al. 2013). These behavioral traits could be visual displays, courtship songs, tactile stimulation or even chemical signals, and comparative studies document that closely related species can differ strongly in these characters. Behavioral traits are often essential for mate recognition and some authors suggest they evolve even faster than morphological structures (Mendelson 2003; Podos et al. 2004; Boul et al. 2007; Podos and Warren 2007; Williams and Mendelson 2010).

Molecular data are a particularly useful tool in reconstructing the spatial and temporal patterns of genetic diversification among taxonomic groups. One particularly fast-evolving marker, the mitochondrial *cytochrome oxidase c subunit I* (*COI*), has been reported to show higher levels of interspecific genetic differentiation compared to other genes in the mitochondrial and nuclear genome (Wenink et al. 1996; Barrowclough et al. 2004). As a result, *COI* sequence data are commonly used for estimating rapid divergence among widespread species (DeSalle et al. 2005; Hebert and Gregory 2005; Meier et al. 2006). This gene has also been used in recent studies to estimate the relative degree of divergence between both morphology and behavior (Puniamoorthy et al. 2009, 2010).

What to expect in this dissertation

The following five chapters represent an integrative study of population divergence and speciation in two dung fly species, *Sepsis punctum* and *Archisepsis diversiformis* (Sepsidae: Diptera). They investigate the role of sexual selection in shaping population level divergence in sexual size dimorphism (Chapters 1 & 2), underlying genetic variation (Chapter 3), intra-specific differences in chemical cues (Chapter 4), and reproductive morphology and mating behavior (Chapters 2 & 5) using a broad range of methods.

Note: The chapters are presented as separate manuscripts, the first two being published already, and hence some parts are inevitably repetitive.

REFERENCES

Andersen, N. M. 1994. The evolution of sexual size dimorphism and mating systems in water striders (Hemiptera: Gerridae). Ecoscience 1:208-214.

Andersson, M. 1994. Sexual selection. Princeton University Press, Princeton, New Jersey.

Arnqvist, G., and I. Danielsson. 1999. Copulatory behavior, genital morphology, and male fertilization success in water striders. Evolution 53:147-156.

Arnqvist, G., and L. Rowe. 2005. Sexual Conflict. Princeton University Press, Princeton.

Badyaev, A. 2002. Growing apart: an ontogenetic perspective on the evolution of sexual size dimorphism. Trends in Ecology & Evolution 17:369-378.

Barrowclough, G. F., J. G. Groth, L. A. Mertz, and R. J. Gutierrez. 2004. Phylogeographic structure, gene flow and species status in blue grouse (Dendragapus obscurus). Molecular Ecology 13:1911-1922.

Blanckenhorn, W. U. 2000. The evolution of body size: What keeps organisms small? Quarterly Review of Biology 75:385-407.

Blanckenhorn, W. U. 2005. Behavioral causes and consequences of sexual size dimorphism. Ethology 111:977-1016.

Blanckenhorn, W. U., R. Meier, and T. Teder. 2007. Rensch's rule in insects: patterns among and within species. Pp. 60-70 *in* D. J. Fairbairn, W. U. Blanckenhorn, and T. Szekely, eds. Sex, size, and gender roles: evolutionary studies of sexual size dimorphism.

Boul, K. E., W. C. Funk, C. R. Darst, D. C. Cannatella, and M. J. Ryan. 2007. Sexual selection drives speciation in an Amazonian frog. Proceedings of the Royal Society B-Biological Sciences 274:399-406.

Cox, R. M., and R. Calsbeek. 2010. Sex-specific selection and intraspecific variation in sexual size dimorphism. Evolution 64:798-809.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Darwin, C. 1871. The Descent of Man, and Selection in Relation to Sex. John Murray, London.

DeSalle, R., M. G. Egan, and M. Siddall. 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philosophical Transactions of the Royal Society B-Biological Sciences 360:1905-1916.

Dobzhansky, T., and E. Mayr. 1944. Experiments on sexual isolation in Drosophila I Geographic strains of Drosophila willistoni. Proceedings of the National Academy of Sciences of the United States of America 30:238-244.

Drovetski, S. V., S. Rohwer, and N. A. Mode. 2006. Role of sexual and natural selection in evolution of body size and shape: a phylogenetic study of morphological radiation in grouse. Journal of Evolutionary Biology 19:1083=1091.

Eberhard, W. G. 1985. Sexual Selection and Animal Genitalia. Eberhard, W. G. Sexual Selection and Animal Genitalia. Xii+244p. Harvard University Press: Cambridge, Mass., USA; London, England. Illus.

Eberhard, W. G. 1996. Female control: Sexual selection by cryptic female choice. Princeton University Press, Princeton.

Emerson, S. B., and R. Ward. 1998. Male secondary sexual characteristics, sexual selection, and molecular divergence in fanged ranid frogs of Southeast Asia. Zoological Journal of the Linnean Society 122:537-553.

Fairbairn, D. J. 1997. Allometry for sexual size dimorphism: Pattern and process in the coevolution of body size in males and females. Annual Review of Ecology and Systematics 28:659-687.

Fairbairn, D. J., W. U. Blanckenhorn, and T. Szekely. 2007. Sex, size and gender roles: evolutionary studies of sexual size dimorphism. . Oxford University Press, London, UK.

Fairbairn, D. J., and R. F. Preziosi. 1996. Sexual selection and the evolution of sexual size dimorphism in the water strider, Aquarius remigis. Evolution 50:1549-1559.

Gröning, J., and A. Hochkirch. 2008. Reproductive interference between animal species. Quarterly Review of Biology 83:257-282.

Hebert, P. D. N., and T. R. Gregory. 2005. The promise of DNA barcoding for taxonomy. Systematic Biology 54:852-859.
Ingram, K. K., T. Laamanen, N. Puniamoorthy, and R. Meier. 2008a. Lack of morphlogical coevolution between male forelegs and female wings in Themira (Sepsidae: Dipera: Insecta). Biological Journal of the Linnean Society 93:227-238.
Kraushaar, U., and W. U. Blanckenhorn. 2002. Population variation in sexual selection and its effect on size allometry in two dung fly species with contrasting sexual size dimorphism. Evolution 56:307-321.
Lindenfors, P., B. S. Tullberg, and M. Biuw. 2002. Phylogenetic analyses of sexual selection and sexual size dimorphism in pinnipeds. Behavioral Ecology and Sociobiology 52:188-193.
Meier, R., S. Kwong, G. Vaidya, and P. K. L. Ng. 2006. DNA Barcoding and Taxonomy in Diptera: a Tale of High Intraspecific Variability and Low Identification Success. Systematic Biology 55:715-728.
Mendelson, T. C. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae : Etheostoma). Evolution 57:317-327.
Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. Trends in Ecology & Evolution 16:364-371.
Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biological Reviews of the Cambridge Philosophical Society 45:525-&.
Parker, G. A. 1979. Sexual selection and sexual conflict. Pp. 123-166 *in* M. S. Blum, and N. A. Blum, eds. Sexual Selection and Reproductive Competition in Insects. Academic, New Jersey.
Parker, G. A. 2006. Sexual conflict over mating and fertilization: an overview. Philosophical Transactions of the Royal Society B-Biological Sciences 361:235-259.
Parker, G. A., and T. Pizzari. 2010. Sperm competition and ejaculate economics. Biological Reviews 85:897-934.
Podos, J., S. K. Huber, and B. Taft. 2004. Bird song: the interface of evolution and mechanism. . Annual Review of Ecology, Evolution, and Systematics 35.
Podos, J., and P. S. Warren. 2007. The evolution of geographic variation in birdsong. Pp. 403-458. Advances in the Study of Behavior, Vol 37.
Preziosi, R. F., and D. J. Fairbairn. 2000. Lifetime selection on adult body size and components of body size in a waterstrider: Opposing selection and maintenance of sexual size dimorphism. Evolution 54:558-566.
Puniamoorthy, N., M. R. B. Ismail, D. S. H. Tan, and R. Meier. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behaviour of 27 species of sepsid flies (Diptera: Sepsidae). Journal of Evolutionary Biology 22:2146-2156.
Puniamoorthy, N., M. Kotrba, and R. Meier. 2010. Unlocking the "Black box": internal female genitalia in Sepsidae (Diptera) evolve fast and are species-specific. Bmc Evolutionary Biology 10.
Puniamoorthy, N., K. F. Y. Su, and R. Meier. 2008. Bending for love: losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae : Diptera). Bmc Evolutionary Biology 8.
Ramos, M., J. A. Coddington, T. E. Christenson, and D. J. Irschick. 2005. Have male and female genitalia coevolved? A phylogenetic analysis of genitalic morphology and sexual size dimorphism in web-building spiders (Araneae : Araneoidea). Evolution 59:1989-1999.
Reuter, M., J. R. Linklater, L. Lehmann, K. Fowler, T. Chapman, and G. D. D. Hurst. 2008. Adaptation to experimental alterations of the operational sex ratio in populations of Drosophila melanogaster. Evolution 62:401-412.
Rowe, L., K. P. Westlake, and D. C. Currie. 2006. Functional significance of elaborate secondary sexual traits and their evolution in the water strider genus Rheumatobates. Canadian Entomologist 138:568-577.

Rugman-Jones, P. F., and P. E. Eady. 2008. Co-evolution of male and female reproductive traits across the Bruchidae (Coleoptera). Functional Ecology 22:880-886.
Seehausen, O., J. J. M. vanAlphen, and F. Witte. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. Science 277:1808-1811.
Serrano-Meneses, M. A., A. Cordoba-Aguilar, V. Mendez, S. J. Layen, and T. Szekely. 2007. Sexual size dimorphism in the American rubyspot: male body size predicts male competition and mating success. Animal Behaviour 73:987-997.
Simmons, L. W. 2001. The evolution of polyandry: an examination of the genetic incompatibility and good-sperm hypotheses. Journal of Evolutionary Biology 14:585-594.
Simmons, L. W. 2005. The evolution of polyandry: Sperm competition, sperm selection, and offspring viability. Pp. 125-146. Annual Review of Ecology Evolution and Systematics.
Simmons, L. W., and D. J. Emlen. 2006. Evolutionary trade-off between weapons and testes. Proceedings of the National Academy of Sciences of the United States of America 103:16346-16351.
Stillwell, R. C., W. U. Blanckenhorn, T. Teder, G. Davidowitz, and C. W. Fox. 2010. Sex Differences in Phenotypic Plasticity Affect Variation in Sexual Size Dimorphism in Insects: From Physiology to Evolution. Pp. 227-245. Annual Review of Entomology.
Takami, Y., and T. Sota. 2007. Rapid diversification of male genitalia and mating strategies in Ohomopterus ground beetles. Journal of Evolutionary Biology 20:1385-1395.
Teuschl, Y., C. Reim, and W. U. Blanckenhorn. 2007. Correlated responses to artificial body size selection in growth, development, phenotypic plasticity and juvenile viability in yellow dung flies. Journal of Evolutionary Biology 20:87-103.
Wenink, P. W., A. J. Baker, H. U. Rosner, and M. G. J. Tilanus. 1996. Global mitochondrial DNA phylogeography of holarctic breeding dunlins (Calidris alpina). Evolution 50:318-330.
Williams, T. H., and T. C. Mendelson. 2010. Behavioral Isolation Based on Visual Signals in a Sympatric Pair of Darter Species. Ethology 116:1038-1049.
Wojcieszek, J. M., and L. W. Simmons. 2012. Evidence for stabilizing selection and slow divergent evolution of male genitalia in a millipede (*Antichirpus variabilis*). Evolution 66:1138-1153.
Zeh, J. A., and D. W. Zeh. 2007. Mate choice by non-virgin females contributes to reproductive isolation between populations of the harlequin beetle-riding pseudoscorpion. Ethology 113:1202-1211.

CHAPTER ONE

Sexual selection accounts for a geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae).

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ABSTRACT

Sexual size dimorphism (SSD) varies widely across and within species. The differential equilibrium model of SSD explains dimorphism as the evolutionary outcome of consistent differences in natural and sexual selection between the sexes. Here we comprehensively examine a unique cross-continental reversal in SSD in the dung fly, *Sepsis punctum*. Using common garden laboratory experiments, we establish that SSD is male-biased in Europe and female-biased in North America. When estimating sexual (pairing success) and fecundity selection (clutch size of female partner) on males under three operational sex ratios (OSR), we find that the intensity of sexual selection is significantly stronger in European vs. North American populations, increasing with male body size and OSR in the former only. Fecundity selection on female body size also increases strongly with egg number and weakly with egg volume, however equally on both continents. Finally, viability selection on body size in terms of intrinsic (physiological) adult lifespan in the laboratory is overall nil and does not vary significantly across all seven populations. Although it is impossible to prove causality, our results confirm the differential equilibrium model of SSD in that differences in sexual selection intensity account for the reversal in SSD in European vs. North American populations, presumably mediating the ongoing speciation process in *Sepsis punctum*.

KEYWORDS

Sepsid flies; body size; natural selection; sexual selection; population differentiation; speciation

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1. INTRODUCTION

Evolutionary biologists largely agree that divergence in sexual dimorphism and mating behavior is frequently driven by sexual selection (Andersson 1994; Arnqvist et al. 2000; Gray and Cade 2000; Panhuis et al. 2001; Boake 2005; Gavrilets and Hayashi 2005; Ritchie 2007). Differences in body size between the sexes, or sexual size dimorphism (SSD), is ubiquitous but variable across the animal kingdom. Species and even populations within a species can differ greatly in the direction and extent of SSD, and there are numerous studies exploring the evolutionary mechanisms underlying this variation (Andersen 1994; Fairbairn 1997; Blanckenhorn 2000; Ding and Blanckenhorn 2002; Drovetski et al. 2006; Blanckenhorn et al. 2007a; Fairbairn et al. 2007; Serrano-Meneses et al. 2007; Stillwell and Fox 2007; Teuschl et al. 2007). It is established that body size affects reproductive success via different mechanisms in the sexes, so the optimal size associated with the maximum fitness often varies for males and females. According to the differential equilibrium model of the evolution of SSD, dimorphism in body size arises when the net effects of sexual and natural selection differ between the sexes (Price 1984; Andersson 1994; Preziosi and Fairbairn 2000; Blanckenhorn 2000). For instance, most mammals and many birds exhibit male-biased SSD, which is primarily attributed to greater mating success of larger males due to male-male competition (via access to territories and/or females) or female choice (Abouheif and Fairbairn 1997; Moore and Wilson 2002). SSD is typically reversed among invertebrates and most ectothermic vertebrates, where female-biased SSD is driven by strong fecundity selection in terms of increased investment in offspring production associated with larger female size (Abouheif and Fairbairn 1997; Blanckenhorn et al. 2007b; Stillwell et al. 2010). Fecundity and sexual selection for larger females or males is presumably held in check by counteracting forces favoring small size in terms of adult and/or juvenile viability or survival, although empirical evidence for these selective processes is far scarcer and often difficult to come by (Blanckenhorn 2000). Additionally, the degree to which the sexes differ in size is also considerably affected by genetic, developmental and phylogenetic constraints (Badyaev 2002; Lindenfors et al. 2002; Ramos et al. 2005; Hu et al. 2010; Tammaru et al. 2010).

Although the above arguments intuitively explain variation in dimorphism among taxa, they are necessarily simplistic and incomplete because the crucial issue is the *relative* strength of sex-specific sexual, fecundity and viability selection in any given species (Price 1984; Arak 1988; Schluter et al. 1991; Andersson 1994; Blanckenhorn 2000). For example, strong sexual selection for large males also regularly occurs in species with smaller males (Andersson 1994; Fairbairn and Preziosi 1994; Fairbairn 1997; Blanckenhorn et al. 1999). In the ideal case, when all the relevant selection pressures

are measured, the differential equilibrium model can generate quantitative predictions about the SSD expected of a given population or species (Arak 1988; Blanckenhorn 2000; Preziosi and Fairbairn 2000; Fairbairn et al. 2007). Therefore the model has to be tested in a micro-evolutionary context by comparing populations of a single species exhibiting variation in dimorphism (e.g. Storz et al. 2001; Schauble 2004; Teder and Tammaru 2005; McGarrity and Johnson 2009; Lyapkov et al. 2010; Yu et al. 2010). However, in most cases intra-specific variation in SSD is slight and quantitative but not qualitatively reversed. We know of only one study documenting albeit minor SSD reversals in some traits but not others in the house finch (Badyaev and Hill 2000). Here we investigate a unique example of strong qualitative reversal in SSD among cross-continental populations of the dung fly *Sepsis punctum* (Fabricius, 1794; Diptera: Sepsidae).

Sepsidae are a family of flies with approximately 320 described species across 36 known genera. Like most insects, sepsid flies generally display female-biased size dimorphism, although examination of museum specimens indicates that in some species SSD is male-biased (Blanckenhorn et al. 2007b). *Sepsis punctum* in particular has a widespread distribution ranging from North America to Europe, North Africa and parts of Asia. It is a generalist that can be found on various types of decaying organic matter, although vertebrate excrements, and cow dung in particular, are its most common breeding substrate (Pont and Meier 2002). Schulz (1999; unpublished doctoral dissertation) first noticed that SSD might be reversed between European and Northern American *Sepsis punctum.* This situation presents the ideal opportunity to test the differential equilibrium model of SSD across replicate cross-continental *Sepsis punctum* populations that vary in both the direction and magnitude of SSD. Using laboratory common garden experiments, we first ascertain whether SSD is indeed male-biased in European and female-biased in American populations. Using standardized quantitative measures of selection (Lande and Arnold 1983; Arnold and Wade 1984a,b), we next estimate (i) adult viability selection on body size in terms of intrinsic (physiological) longevity, (ii) fecundity selection on female body size in terms of clutch and egg size, and (iii) sexual and fecundity selection on male body size in terms of male mating success and the number of eggs of his mate (assortative mating). We estimate sexual selection in population cages at three operational sex ratios (OSR), as a function of which competition for mates and consequently the intensity of sexual selection is expected to increase (Bonduriansky 2001). According to the equilibrium model of SSD, we expect that in the European populations of *S. punctum* the intensity of sexual selection on male size should be greater than the intensity of fecundity selection on female size, whereas this should be reversed in North America; in other words, continental differences in sexual selection on

male size should be large compared to continental differences in fecundity selection on female size and in viability selection on male and female size, which should be small or non-existent.

2. METHODS

2.1. Population sampling and fly culture maintenance

We sampled four European *S. punctum* populations from Nyköping, Sweden (SE: 58.67°N, 16.94°E), Berlin, Germany (DE: 52.45°N, 13.28°E), Vienna, Austria (A: 48.20°N, 16.36°E) and Zürich, Switzerland (CH: 47.40°N, 8.55°E), and three North American populations from Davis, California (CA: 38.54°N, -121.75°W), Athens, Georgia (GA: 33.96°N, -83.38°E) and Manhattan, New York (NY: 40.78°N, -73.96°E). Wild caught females were brought to the laboratory and used to establish stock cultures of multiple (10 to 20) replicate lines per population that were housed in separate plastic containers and regularly supplied with fresh cow dung, sugar and water *ad libitum*.

2.2. Common garden experiments

We conducted laboratory common garden experiments to ascertain patterns of SSD among the European and North American populations. We allowed mated females, housed in replicate group containers per population, to oviposit in pots of fresh cow dung for two to three hours. We then reared the offspring in groups in abundant cow dung in a climate chamber at standardized 24^{o}C, 60% humidity and 14 h light cycle, measured the development time and head width of emergent flies as a standard index of body size. This method of using laboratory lines instead of wild caught females removes confounding environmental variation influencing phenotypic body size, establishing that the body size differentiation is indeed heritable.

2.3. Testing the differential equilibrium model

2.3.1. Adult viability (i.e. intrinsic longevity) selection:

Viability selection on males and females is affected by multiple extrinsic factors such as parasitism, predation, thermoregulation, food availability, etc. as well as by intrinsic physiological and genetic factors (reflecting ageing). Estimation of juvenile or adult mortality as a function of body size in the wild in small mobile insects is essentially impossible. Instead we tested whether there are size- and sex-dependent differences in intrinsic adult longevity between European and North American populations as a function of body size under laboratory conditions in population cages (cf. Blanckenhorn et al. 1999). We provided stock cultures with varying amounts of dung to generate a range of phenotypic body sizes, and reared the offspring under the standard conditions mentioned earlier. The emerging flies were individually sexed under a microscope within

12 hours of eclosion and set up under two different 'housing' treatments (Teuschl et al. 2010): males only and females only (i.e. two treatments per population; five replicate containers per treatment; approx. 18-20 individual flies per container). Each container was provided with fresh dung, sugar and water *ad libitum.* We monitored all 70 containers and more than 1300 individuals daily for adult mortality. Dead flies were removed every day, scored for adult lifespan and measured for body size (head width).

2.3.2. Fecundity selection

To estimate fecundity selection, we randomly selected 30 – 60 once mated females of various body sizes from the stock lines, set them up individually in glass vials, provided them with fresh dung and counted their first (and sometimes additionally their second) clutch sizes, which is good proxy for life-time fecundity in the study species (Puniamoorthy unpublished data). Since investment in offspring production can also be affected by the amount of resources invested in each egg, we additionally measured the average egg volume of 5 eggs in each clutch for each female in all seven populations. Every female was frozen afterwards and measured for body size (head width).

2.3.3. Sexual selection: Male mating success

For each population, we supplied stock lines with two pots of fresh dung each. To generate individuals of varying sizes, one dish was removed after two hours (no larval competition) whilst the other was left overnight (competition). These dung dishes were subsequently placed into larger plastic containers and housed in climate chambers at 24 ^{o}C. Emerging flies were sexed within 24 hours of eclosion and thereafter housed separately in single-sex group containers with dung, sugar and water. We waited three to four days to ensure sexual maturity and then conducted mating trials with randomly assembled virgin flies in population cages at three operational sex ratios (OSR): 5 males plus 5 females (OSR = 1), 10 males plus 5 females (OSR = 2), and 20 males plus 5 females (OSR = 4). There were 4 – 5 replicates per OSR per population. Females always entered the population container first, which was equipped with water and sugar and some fresh dung; the males were added later. We tracked which male copulated with which female by isolating the mating pairs from the singletons. Each group trial lasted for a maximum of two hours after which all individuals (both mated and unmated) were measured for body size. From these data male sexual and fecundity selection differentials could be calculated.

In this study, since we were only interested in instantaneous pairing success, we did not allow for multiple mating. Early field observations of sepsid flies note that although male densities at a dung pat can rise up to 500 individuals in the first few minutes of the dung

dropping, this number decreases drastically within the first 30 minutes (Hammer 1941). In fact, Parker (1972a, b) additionally showed that in *S. cynipsea,* the highest female arrival, oviposition and capture rates occur within ten minutes of the dropping and declines sharply after that. Copulation in *S. punctum* usually lasts approximately 20-30 minutes (Puniamoorthy, pers. obs.), during which time males are not available for re-mating. Hence, given that dung pats in nature become unattractive as oviposition sites quickly, multiple mating at the same dropping is relatively unlikely, so we believe our experimental setup simulates nature rather well.

2.4. Statistical Analysis

We used standardized regression methods to generate univariate linear selection differentials to assess the intensity of adult viability, female fecundity and male sexual and fecundity selection on (adult) body size (Lande and Arnold 1983; Arnold and Wade 1984a,b). In general, for each population and replicate container we produced standardized *z*-scores for body size (head width) by subtracting the sample mean from each value and dividing the difference by the standard deviation: $z_i = (x_i - \bar{x})/SD_x$. Relative fitness was calculated as the absolute fitness component (i.e. adult longevity, female clutch and egg size, and male pairing success (1 or 0) or the body size of his female partner) divided by the population or container mean fitness (Arnold and Wade 1984b). We used models of relative fitness on z-scored body size $w = c + \beta_1 z$ to estimate univariate linear selection differentials.

To estimate viability selection, we regressed adult longevity on standardized body size, separately for the sexes and the replicate containers within populations. This yielded one viability selection estimate per replicate container. All 5 estimates per population were then averaged, yielding a corresponding confidence interval.

For female fecundity selection, we regressed relative clutch size or relative egg volume on standardized female body size. Selection coefficients of consecutive selection episodes are additive because fitness components are cumulative and hence multiplicative (Arnold and Wade 1984b). Thus, we can easily compute a female fecundity selection differential subsuming clutch and egg size. This yielded one fecundity selection differential per population with its appropriate standard error (or confidence interval) derived from regression.

A male's reproductive success is affected by both his mating success and the fecundity of his mate, which in turn depends on her body size (as above). We estimated sexual selection differentials based on mating success (males that copulated vs. those that did

not) separately for each replicate container. Additionally, we regressed relative female body size (being proportional to her fecundity) on standardized male body size. Adding (i.e. subsuming) both yielded the male fecundity selection differentials, one estimate per replicate container for all populations and OSRs, which were then averaged, yielding a corresponding confidence interval (see e.g. Blanckenhorn et al. 1999 for further details on these methods).

The above procedure describes calculation of the selection differential estimates. Significance testing, for all fitness components, was performed using the full models including continent, population nested within continent, replicate nested within population within continent (not applicable for female fecundity selection), and OSR (sexual selection only) as fixed or random factors and body size as a continuous covariate, including all relevant interaction terms. Variation in selection in all cases is established by significant factor by body size interactions. All analyses were done using the software SPSS version 10.0 (Norušis 2000).

3. RESULTS

3.1. Common garden experiments

SSD is clearly reversed comparing the two continents, with populations displaying male-biased SSD in Europe and female-biased SSD in North America (Figure 1; continent by sex interaction: $F_{1,5} = 27.88$, $P = 0.003$). Further, European flies are on average larger than North American flies and take longer to develop (Table 1; Body size: $F_{1,5} = 12.77$, $P = 0.016$; Development time: $F_{1,5} = 5.46$, $P = 0.067$; continent by sex interaction: $F_{1,5} = 10.22$, $P = 0.023$).

3.2. Testing the differential equilibrium model

3.2.1. Adult viability (i.e. intrinsic longevity) selection

Adult viability was overall slightly positively related with body size ($F_{1,1228} = 3.98$, $P = 0.046$), thus implying no counterselection against large body size (contrary to expectation: cf. Blanckenhorn 2000). This effect could largely be attributed to the Austrian males and the New York population (both sexes); all other populations showed no effect whatsoever of body size on adult longevity (Table 2; mean level and range indicated in Figure 2). Standardized adult viability selection coefficients for males range between -0.048 ± 0.105 (95% CI) for the Swedish population and +0.157 ± 0.493 for the Austrian population; for females the range is from -0.010 ± 0.102 (95% CI) for the Georgian population and +0.079 ± 0.246 for the Austrian population (Table 2). There were strong systematic differences between the sexes in longevity (females living longer on average; $F_{1,1228} = 16.86$, $P < 0.001$), some unsystematic variation among populations

($F_{5,28}$ = 2.55, P = 0.050), but no significant difference between the continents ($F_{1,28}$ = 0.04, P = 0.847; corresponding sex by factor interactions also n.s.). Viability selection for body size consequently was largely nil and did not vary systematically between the continents, the sexes, or the populations (all corresponding factor by body size interactions P > 0.1, except the three-way sex by population by body size interaction: $F_{5,1187}$ = 3.33, P = 0.005).

3.2.2. Fecundity selection

Larger females lay larger clutches in all populations (overall strong main effect of body size: $F_{1,317}$ = 610.58, P < 0.0001; Table 2). Standardized female fecundity selection coefficients based on clutch size range between 0.169 ± 0.057 (95% CI) for the California population and 0.343 ± 0.047 for the New York population (mean and range indicated in Figure 2; Table 2). Clutch size varied among populations within continents ($F_{5,317}$ = 15.63, P = 0.001), but not between continents ($F_{1,5}$ = 0.63, P = 0.427). Crucially, fecundity selection differentials on body size (based on clutch size) did not vary among populations within continents (population by body size interaction: $F_{5,317}$ = 1.35, P = 0.244) or among continents (continent by body size interaction: $F_{1,317}$ = 1.17, P = 0.280).

Overall, larger females also laid larger eggs (main effect of body size: $F_{1,175}$ = 15.85, P < 0.001; Table 2), but the relationship with body size was much weaker. Corresponding standardized female fecundity selection coefficients based on (cube-root-transformed) egg volume range between 0.002 ± 0.010 (95% CI) for the Swedish population and 0.020 ± 0.015 for the New York population. We had egg volume data for about half of the clutches treated above, which varied unsystematically among populations within continents ($F_{5,175}$ = 6.08, P < 0.001), but not among continents ($F_{1,5}$ = 0.96, P = 0.443). However, when tested against the global error, eggs were significantly smaller in North America than in Europe after controlling for body size ($F_{1,175}$ = 6.01, P = 0.015). Nevertheless, fecundity selection on body size based on egg volume did not vary among populations within continents (population by body size interaction: $F_{5,175}$ = 0.49, P = 0.781) or among continents (continent by body size interaction: $F_{1,175}$ = 2.10, P = 0.148).

3.2.3. Sexual selection

In the European populations, 42 out of the 48 replicate sexual selection differentials based on pairing success were positive, indicating strong sexual selection for larger male body size. Further, sexual selection for large males intensified with increasing OSR and with body size, supporting Rensch's rule (Figure 2). Sexual selection differentials for the

American populations were also generally positive (27 out of 36) albeit lower, but there was no clear pattern of increased selection with OSR (Figure 2; Table 2). The full (logistic) general linear model consequently indicated overall strong positive effects of body size (head width) on pairing success ($F_{1,930}$ = 22.23, P < 0.001), a significant interaction of continent and OSR ($F_{2,22}$ = 3.34, P = 0.044), and, most importantly, a significant OSR-by-continent-by-body size interaction ($F_{2,930}$ = 3.87, P = 0.021). The latter demonstrates variation in sexual selection on body size among the continents and the three OSR treatments.

Selection differentials reflecting assortative mating by size given pairing and hence the fecundity of the female partner were weak in comparison and did not vary significantly, ranging from -0.018 to 0.123; nevertheless, on average these added to the sexual selection differentials based on pairing success, making the combined male fecundity selection differentials even more positive across all populations and OSRs (73 out of 84) (Table 2).

Figure 1. Sexual body size dimorphism in 7 cross-continental populations of the dung fly *Sepsis punctum* (Sample size: Europe= 498, N.America= 618).

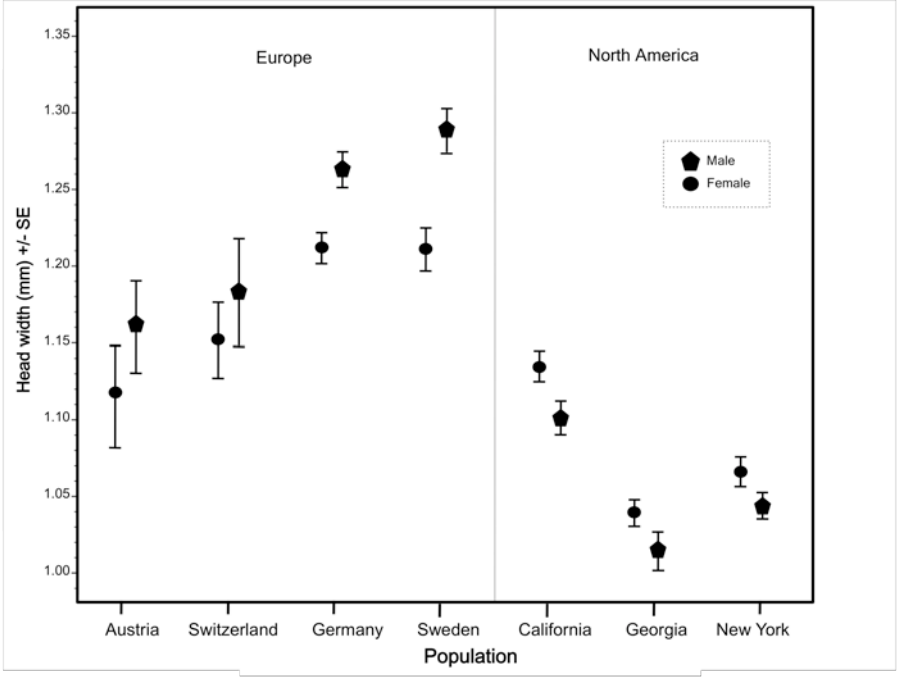

Figure 2. Mean fecundity (sexual) selection intensity on male body size in 7 cross-continental populations of the black scavenger fly *Sepsis punctum* at three operational sex ratios (OSR). White, grey and black boxes show selection intensity increases with OSR (i.e. male competition). The (equal) levels of fecundity selection on female body size (light grey bars; confidence limits) and of adult viability selection (dark grey bars; confidence limits) on female and male body size do not differ significantly between the continents.

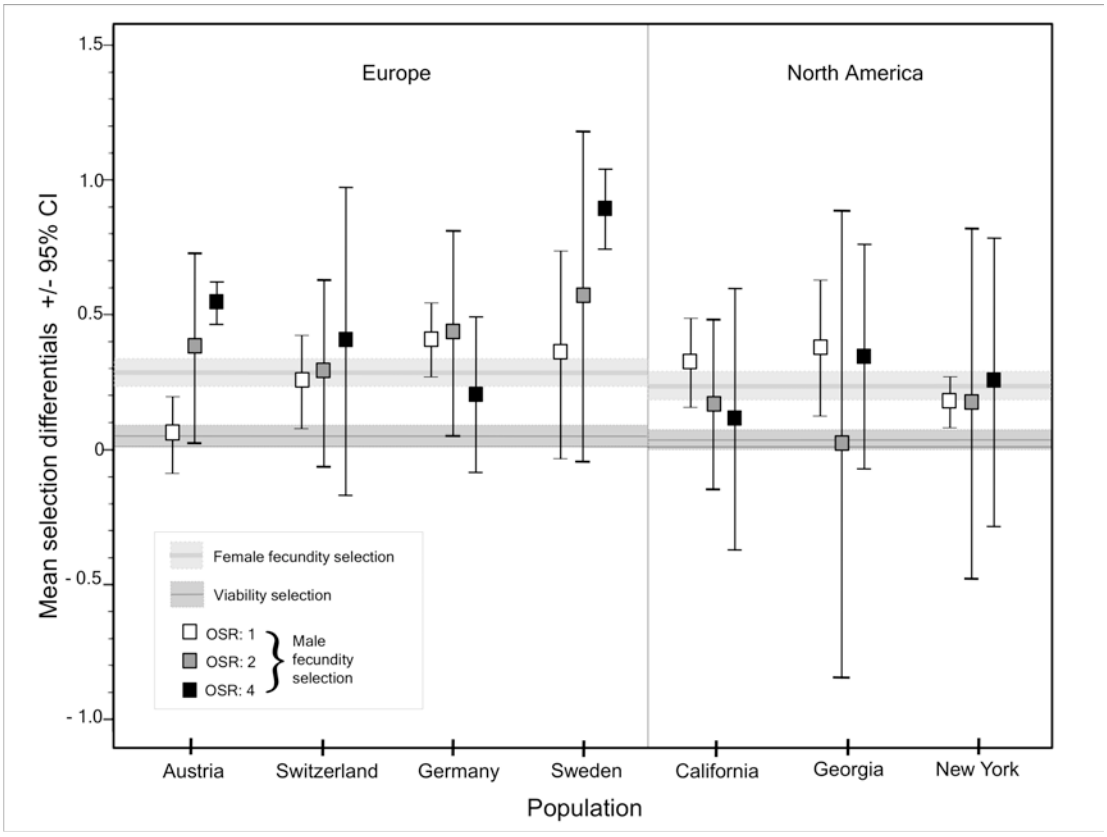

Table 1. Population mean (± SE) for body size, development time, adult longevity, female clutch size, egg volume and male pairing success (under different OSRs) (Sample size, n).

			Common garden			***Adult viability***			
	Population	Sex	Head width (mm)	Development time (days)	n	"Housing" treatment	Head width (mm)	Lifespan (days)	n
Europe	Austria	Male	1.16 ± 0.07	14.25 ± 0.65	28	Male only	1.08 ± 0.07	69.16 ± 41.82	91
		Female	1.12 ± 0.10	13.54 ± 0.95	23	Female only	1.06 ± 0.05	77.24 ± 35.19	93
	Germany	Male	1.26 ± 0.04	14.45 ± 0.55	80	Male only	1.05 ± 0.13	48.23 ± 35.31	100
		Female	1.21 ± 0.03	13.54 ± 0.54	100	Female only	0.98 ± 0.13	62.15 ± 30.96	99
	Switzerland	Male	1.18 ± 0.14	15.40 ± 0.46	63	Male only	0.94 ± 0.10	74.55 ± 42.37	93
		Female	1.15 ± 0.09	14.91 ± 0.86	105	Female only	0.97 ± 0.10	71.42 ± 40.73	85
	Sweden	Male	1.29 ± 0.06	14.6 ± 0.88	47	Male only	1.36 ± 0.13	51.97 ± 27.00	98
		Female	1.21 ± 0.05	14.27 ± 0.84	52	Female only	1.07 ± 0.10	65.42 ± 31.60	100
North America	California	Male	1.10 ± 0.03	13.93 ± 0.72	83	Male only	0.97 ± 0.04	68.70 ± 36.43	91
		Female	1.14 ± 0.03	13.72 ± 1.81	127	Female only	1.00 ± 0.05	59.66 ± 34.27	87
	Georgia	Male	1.02 ± 0.04	12.29 ± 0.86	66	Male only	0.84 ± 0.10	55.93 ± 33.41	98
		Female	1.05 ± 0.03	12.55 ± 1.00	67	Female only	0.87 ± 0.09	78.76 ± 34.66	101
	New York	Male	1.05 ± 0.03	14.34 ± 0.78	112	Male only	0.98 ± 0.04	51.30 ± 24.37	79
		Female	1.07 ± 0.03	14.69 ± 0.84	163	Female only	1.00 ± 0.05	60.22 ± 33.08	102

		Female fecundity					***Male mating success***				
	Population	Head width (mm)	First clutch	n	Egg volume	n	OSR	Head width (mm) Paired	n	Unpaired	n
Europe	Austria	1.02 ± 0.13	81.03 ± 2.54	33	0.25 ± 0.01	33	1	1.09 ± 0.16	22	1.09 ± 0.18	18
							2	1.02 ± 0.17	19	0.93 ± 0.15	22
							4	1.04 ± 0.19	22	0.92 ± 0.17	58
	Germany	1.09 ± 0.11	59.61 ± 23.27	56	0.24 ± 0.01	19	1	1.08 ± 0.54	20	1.04 ± 0.07	20
							2	1.09 ± 0.05	20	1.04 ± 0.06	20
							4	1.07 ± 0.06	19	1.05 ± 0.07	61
	Switzerland	0.98 ± 0.13	69.83 ± 25.00	57	0.24 ± 0.01	20	1	1.18 ± 0.09	22	1.14 ± 0.07	18
							2	1.18 ± 0.08	20	1.15 ± 0.07	20
							4	1.18 ± 0.09	21	1.14 ± 0.07	60
	Sweden	1.06 ± 0.14	89.80 ± 27.90	30	0.26 ± 0.01	29	1	1.13 ± 0.18	21	1.02 ± 0.15	19
							2	1.16 ± 0.12	20	0.99 ± 0.15	20
							4	1.21 ± 0.10	20	1.00 ± 0.17	60
North America	California	1.03 ± 0.10	68.40 ± 18.00	47	0.24 ± 0.01	34	1	1.01 ± 0.08	21	0.99 ± 0.09	19
							2	1.01 ± 0.08	18	0.98 ± 0.09	22
							4	1.00 ± 0.08	16	1.00 ± 0.09	59
	Georgia	0.94 ± 0.07	71.79 ± 14.52	46	0.24 ± 0.01	40	1	0.90 ± 0.06	27	0.82 ± 0.09	23
							2	0.92 ± 0.07	15	0.92 ± 0.08	25
							4	0.96 ± 0.03	18	0.93 ± 0.07	58
	New York	0.95 ± 0.13	64.42 ± 24.94	58	0.24 ± 0.01	16	1	1.02 ± 0.03	19	1.00 ± 0.04	19
							2	0.99 ± 0.03	14	0.99 ± 0.04	28
							4	1.00 ± 0.04	13	0.99 ± 0.04	66

Table 2. Univariate selection differentials (mean ± 95% CI) for adult viability selection ($ß_{VS}$), female fecundity selection ($ß_{FS}$), male sexual selection ($ß_{SexS}$) and male fecundity selection ($ß_{mFS}$).

			Adult viability	***Female fecundity***	***Male reproductive success***		
	Population	"Housing" treatment	$ß_{VS}$	$ß_{FS}$	OSR	$ß_{SexS}$	$ß_{mFS}$
Europe	Austria	Female only	0.079 ± 0.246	0.248 ± 0.127	1	0.013 ± 0.177	0.040 ± 0.177
		Male only	0.157 ± 0.493		2	0.324 ± 0.436	0.363 ± 0.436
					4	0.438 ± 0.095	0.532 ± 0.095
	Germany	Female only	0.020 ± 0.148	0.326 ± 0.053	1	0.311 ± 0.165	0.428 ± 0.165
		Male only	-0.030 ± 0.105		2	0.427 ± 0.475	0.418 ± 0.475
					4	0.208 ± 0.356	0.190 ± 0.356
	Switzerland	Female only	0.013 ± 0.223	0.291 ± 0.057	1	0.179 ± 0.215	0.302 ± 0.215
		Male only	0.009 ± 0.163		2	0.206 ± 0.422	0.219 ± 0.422
					4	0.388 ± 0.698	0.389 ± 0.698
	Sweden	Female only	0.026 ± 0.127	0.263 ± 0.063	1	0.306 ± 0.474	0.340 ± 0.474
		Male only	-0.047 ± 0.020		2	0.521 ± 0.756	0.557 ± 0.756
					4	0.876 ± 0.178	0.881 ± 0.178
North America	California	Female only	0.016 ± 0.165	0.169 ± 0.057	1	0.260 ± 0.198	0.310 ± 0.198
		Male only	-0.004 ± 0.157		2	0.163 ± 0.385	0.154 ± 0.385
					4	0.117 ± 0.598	0.099 ± 0.598
	Georgia	Female only	-0.010 ± 0.102	0.170 ± 0.033	1	0.329 ± 0.393	0.362 ± 0.393
		Male only	0.032 ± 0.181		2	0.015 ± 1.064	0.007 ± 1.064
					4	0.342 ± 0.511	0.330 ± 0.511
	New York	Female only	0.025 ± 0.131	0.343 ± 0.047	1	0.053 ± 0.120	0.163 ± 0.120
		Male only	0.068 ± 0.338		2	0.155 ± 0.799	0.157 ± 0.799
					4	0.229 ± 0.659	0.237 ± 0.659

4. DISCUSSION

We have shown here that a unique reversal in sexual size dimorphism between European and Northern American populations of the black scavenger fly *Sepsis punctum* is associated with, and presumably mediated by, substantial differences in the strength of positive sexual selection on males. As a result, European flies are larger than North American flies and SSD is male-biased and stronger, in agreement with Rensch's rule (Fairbairn 1997; Blanckenhorn et al. 2007b; Fairbairn et al. 2007). European females are also larger than North American females despite no differences in fecundity selection on female size, but this can be expected due to a genetic correlation in body size between the sexes alone (Fairbairn 1997). In European (but not North American) populations, sexual selection also increased with the degree of male-male competition for females (i.e. the operational sex ratio: OSR), as expected by sexual selection theory (Bonduriansky 2001). This outcome confirms the differential equilibrium model of the evolution of SSD (Andersson 1994; Preziosi and Fairbairn 2000; Blanckenhorn 2000).

We emphasize that while we were able to show an association between sexual selection intensity and SSD (and probably mating system) evolution in accordance with the differential equilibrium model, such evidence must remain correlational as we cannot reconstruct the causality of evolutionary events. This is because evolutionary shifts in mating behaviors and the mating system are expected to be rapid and intimately associated with changes in sexual selection intensity, ultimately affecting the evolution of body size and SSD (Ding and Blanckenhorn 2002).

We also emphasize that although we considered three major fitness components (viability, fecundity, and sexual selection), comprehensive treatment of all relevant aspects of selection affecting SSD evolution, let alone in the field, is virtually impossible in any single species (Blanckenhorn 2000). In particular, we did not assess juvenile viability selection on body size, which in animals with complex life cycles such as insects is unattainable because larval and adult body size traits cannot easily be compared and individuals that die before adulthood cannot be measured (Blanckenhorn et al. 1999). One of the main mechanisms selecting against large body size occurs because individuals often grow for longer time to become larger, which increases cumulative mortality (Blanckenhorn 2000, 2007; Blanckenhorn et al. 2007a). And indeed, European *S. punctum* have longer development times than North American ones and the sex difference in development time differs between continents (Table 1). However, because the differences in absolute time are small (Table 1), it is doubtful that juvenile viability selection against long development fully compensates the much stronger sexual selection for large male size in European flies (cf. Blanckenhorn 2007). Furthermore,

assessment of intrinsic (i.e. physiological) adult viability in the laboratory, as done here, does not necessarily reflect extrinsic adult viability in the field. Moreover, assessing female fecundity selection in the laboratory is a limited approximation of reproductive output in the field (Clutton-Brock 1988). Nevertheless, given no relationship of intrinsic longevity (lifespan) with body size here, we have confidence in our estimates.

Recent comparative studies have highlighted the rapid divergence in sexual dimorphisms and mating behavior in sepsid flies (Puniamoorthy et al. 2008; Puniamoorthy et al. 2009; Tan et al. 2010). There have also been very early reports of interesting courtship behavior in sepsid flies (Hammer 1941; Hafez 1948; Parker 1972a, b; Mangan 1976). In *S. punctum*, the cross-continental differences in SSD documented here are accompanied by stark differences in the mating system (not treated in detail here; Schulz 1999, unpublished doctoral dissertation). North American populations display pre-copulatory courtship behavior in form of vigorous shaking of the male abdomen when approaching the female, a behavior that is absent in the European populations (Puniamoorthy et al., unpublished data). In contrast, European males show no distinct pre-copulatory courtship but instead scramble and/or contest competition among males, as evident by frequent male-male mountings and common 'take-overs' where a male displaces another mounted male (Parker 1972b; Zerbe 1993). In fact, our ongoing studies indicate that European females also re-mate more readily, whereas North American females re-mate very rarely (Puniamoorthy et al., unpublished data; cf. Teuschl and Blanckenhorn 2007). More detailed, in-depth behavioral studies of the systematic mating system differences between the continents should further help explain the reversal to male-biased SSD in Europe. Although the genetic distance between North American and European *S. punctum* is almost 3% (based on the DNA barcoding gene: R. Meier et al. unpublished data), European and North American flies readily hybridize and produce viable offspring (Schulz 1999; Puniamoorthy et al., unpublished data).

An increasing number of studies have documented considerable intra-specific variation in SSD, usually in response to environmental, latitudinal or even altitudinal clines (e.g. Badyaev and Hill 2000; Teder and Tammaru 2005; Fox and Czesak 2006; Stillwell and Fox 2007; Liu et al. 2010; Hu et al. 2011). Most of these studies treated (quantitative) variation merely in the magnitude of SSD. Our study is a unique exception in that we phenomenologically tested the differential equilibrium model of the evolution of SSD in a species showing strong qualitative variation in dimorphism. We could confirm the model by showing that sexual selection on male body size in *S. punctum* is consistently stronger in European than in North American populations, while fecundity selection acting on female body size and adult viability selection are weaker and not different

between the continents. Unpublished molecular data by R. Meier and colleagues in Singapore (cf. Su et al. 2008) suggest that the SSD and mating system of North American *S. punctum* is the ancestral state as, like many invertebrates, most sepsid species display female-biased SSD. The male-biased SSD in European *S. punctum* populations is therefore presumably secondarily evolved due to sexual selection in association with a change in the mating system, as predicted by theory (Andersson 1994; Fairbairn 1997; Bonduriansky 2001; Ding and Blanckenhorn 2002).

5. REFERENCES

Abouheif, E., and D. J. Fairbairn. 1997. A comparative analysis of allometry for sexual size dimorphism: assessing Rensch's rule. American Naturalist 149:540-562.

Andersen, N. M. 1994. The evolution of sexual size dimorphism and mating systems in water striders (Hemiptera: Gerridae). Ecoscience 1:208-214.

Andersson, M. 1994. Sexual selection. Princeton University Press, Princeton, New Jersey.

Arak, A. 1988. Sexual size dimorphism in body size - A model and a test. Evolution 42:820-825.

Arnold, S. J., and M. J. Wade. 1984a. On the measurement of natural selection and sexual selection - Applications. Evolution 38:720-734.

Arnold, S. J., and M. J. Wade. 1984b. On the measurement of natural selection and sexual selection - Theory. Evolution 38:709-719.

Arnqvist, G., M. Edvardsson, U. Friberg, and T. Nilsson. 2000. Sexual conflict promotes speciation in insects. Proceedings of the National Academy of Sciences 97:10460-10464.

Badyaev, A. 2002. Growing apart: an ontogenetic perspective on the evolution of sexual size dimorphism. Trends in Ecology & Evolution 17:369-378.

Badyaev, A. V., and G. E. Hill. 2000. The evolution of sexual dimorphism in the house finch. I. Population divergence in morphological covariance structure. Evolution 54:1784-1794.

Blanckenhorn, W. U. 2000. The evolution of body size: What keeps organisms small? Quarterly Review of Biology 75:385-407.

Blanckenhorn, W. U. 2007. Case studies of the differential equilibrium hypothesis of sexual size dimorphism in dung flies. In *Sex, Size and Gender Roles. Evolutionary Studies of Sexual Size Dimorphism* (ed. by D. J. Fairbairn, W. U. Blanckenhorn & T. Szekely), Oxford University Press, pp. 106-114.

Blanckenhorn, W. U., A. F. G. Dixon, D. J. Fairbairn, M. W. Foellmer, P. Gibert, K. van der Linde, R. Meier, S. Nylin, S. Pitnick, C. Schoff, M. Signorelli, T. Teder, and C. Wiklund. 2007a. Proximate causes of Rensch's rule: Does sexual size dimorphism in arthropods result from sex differences in development time? American Naturalist 169:245-257.

Blanckenhorn, W. U., U. R. S. Kraushaar, Y. Teuschl, and C. Reim. 2004. Sexual selection on morphological and physiological traits and fluctuating asymmetry in the black scavenger fly Sepsis cynipsea. Journal of Evolutionary Biology 17:629-641.

Blanckenhorn, W. U., R. Meier, and T. Teder. 2007b. Rensch's rule in insects: patterns among and within species. Pp. 60-70 *in* D. J. Fairbairn, W. U. Blanckenhorn, and T. Szekely, eds. Sex, size, and gender roles: evolutionary studies of sexual size dimorphism.

Blanckenhorn, W. U., C. Morf, C. Muhlhauser, and T. Reusch. 1999. Spatiotemporal variation in selection on body size in the dung fly Sepsis cynipsea. Journal of Evolutionary Biology. May, 1999; 12:563-576.

Boake, C. R. B. 2005. Sexual selection and speciation in Hawaiian Drosophila. Behavior Genetics 35:297-303.

Bonduriansky, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. Biological Reviews 76:305-339.

Clutton-Brock T H, Albon S D, Guiness F E. 1988. Reproductive success in male and female deer. Pages 325-343 in Reproductive Success, edited by T H Clutton-Brock. Chicago: University of Chicago Press.

Ding, A., and W. U. Blanckenhorn. 2002. The effect of sexual size dimorphism on mating behaviour in two dung flies with contrasting dimorphism. Evolutionary Ecology Research 4:259-273.

Drovetski, S. V., S. Rohwer, and N. A. Mode. 2006. Role of sexual and natural selection in evolution of body size and shape: a phylogenetic study of morphological radiation in grouse. Journal of Evolutionary Biology 19:1083=1091.

Eberhard, W. G. 2001. Species-specific genitalic copulatory courtship in sepsid flies (Diptera, Sepsidae, Microsepsis) and theories of genitalic evolution. Evolution 55:93-102.

Eberhard, W. G. 2002. Physical restraint or stimulation? The function(s) of the modified front legs of male Archisepsis diversiformis (Diptera, Sepsidae). Journal of Insect Behavior 15:831-850.

Fairbairn, D. J. 1997. Allometry for sexual size dimorphism: Pattern and process in the coevolution of body size in males and females. Annual Review of Ecology and Systematics 28:659-687.

Fairbairn, D. J., W. U. Blanckenhorn, and T. Szekely. 2007. Sex, size and gender roles: evolutionary studies of sexual size dimorphism. Oxford University Press, London, UK.

Fairbairn, D. J., and R. F. Preziosi. 1994. Sexual selection and the evolution of allometry for sexual size dimorphism in the water strider, *Aquarius remigis*. American Naturalist 144:101-118.

Fox, C. W., and M. E. Czesak. 2006. Selection on body size and sexual size dimorphism differs between host species in a seed-feeding beetle. Journal of Evolutionary Biology 19:1167-1174.

Gavrilets, S., and T. I. Hayashi. 2005. Speciation and sexual conflict. Evolutionary Ecology 19:167-198.

Gray, D. A., and W. H. Cade. 2000. Sexual selection and speciation in field crickets. Proceedings of the National Academy of Sciences of the United States of America 97:14449-14454.

Hafez, M. 1948. Ecological and biological observations on some coprophagous Sepsidae (Diptera). Proc. R. Entomol. Soc. London 23:99-103.

Hammer, O. 1941. Biological and ecological investigations on flies associated with pasturing cattle and their excrement. Vidensk. Medd. Dansk Naturhist. Foren. 105:141-394.

Hu, Y. W., Y. J. Xie, F. Zhu, C. B. Wang, and C. L. Lei. 2010. Variation in sexual size dimorphism among populations: testing the differential-plasticity hypothesis. Entomologia Experimentalis Et Applicata 137:204-209.

Hu, Y. W., X. Yuan, and C. L. Lei. 2011. Sexual size dimorphism decreases with temperature in a blowfly, Chrysomya megacephala (Fabricius) (Diptera: Calliphoridae). Ecological Entomology 36:111-115.

Ingram, K. K., T. Laamanen, N. Puniamoorthy, and R. Meier. 2008. Lack of morphlogical coevolution between male forelegs and female wings in Themira (Sepsidae: Dipera: Insecta). Biological Journal of the Linnean Society 93:227-238.

Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. Evolution 37:1210-1226.

Lindenfors, P., B. S. Tullberg, and M. Biuw. 2002. Phylogenetic analyses of sexual selection and sexual size dimorphism in pinnipeds. Behavioral Ecology and Sociobiology 52:188-193.

Liu, X. A., Y. M. Li, and M. McGarrity. 2010. Geographical variation in body size and sexual size dimorphism of introduced American bullfrogs in southwestern China. Biological Invasions 12:2037-2047.

Lyapkov, S. M., V. G. Cherdantsev, and E. M. Cherdantseva. 2010. Geographic variation of sexual dimorphism in the moor frog (Rana arvalis) as a result of differences in reproductive strategies. Zhurnal Obshchei Biologii 71:337-358.

Mangan, R. L. 1976. *Themira athabasca* n. sp. (Diptera: Sepsidae) with a revised key to North American *Themira* and notes on the sexual morphology of sympatric species. Ann. Entomol. Soc. Am. 69:1024-1028.

Martin, O. Y., and D. J. Hosken. 2003. Copulation reduces male but not female longevity in Saltella sphondylli (Diptera: Sepsidae). Journal of Evolutionary Biology 17:357-362.

McGarrity, M. E., and S. A. Johnson. 2009. Geographic trend in sexual size dimorphism and body size of Osteopilus septentrionalis (Cuban treefrog): implications for invasion of the southeastern United States. Biological Invasions 11:1411-1420.

Moore, S. L., and K. Wilson. 2002. Parasites as a viability cost of sexual selection in natural populations of mammals. Science (Washington D C) 297:2015-2018.

Mühlhäuser, C., and W. U. Blanckenhorn. 2004. The quantitative genetics of sexual selection in the dung fly *Sepsis cynipsea*. Behaviour 141:327-341.

Norušis M. J. 2000. SPSS professional statistics 10.0. Chicago.

Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. Trends in Ecology & Evolution 16:364-371.

Parker, G. A. 1972a. Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera: Sepsidae) I. A preliminary analysis of the reproductive strategy and its associated behaviour patterns. Behaviour 41:172-206.

Parker, G. A. 1972b. Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera: Sepsidae) II. The significance of the precopulatory passive phase and emigration. Behaviour 41:242-250.

Pont, A. C., and R. Meier. 2002. The Sepsidae (Diptera) of Europe. Fauna Entomologica Scandinavica 37:1-221.

Preziosi, R. F., and D. J. Fairbairn. 2000. Lifetime selection on adult body size and components of body size in a waterstrider: Opposing selection and maintenance of sexual size dimorphism. Evolution 54:558-566.

Price, T. D. 1984: The evolution of sexual dimorphism in Darwin's Finches. Am. Nat. 123, 500-518.

Puniamoorthy, N., K. Su Feng Yi, and R. Meier. 2008. Bending for love: losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae: Diptera). BMC Evolutionary Biology 8:155.

Puniamoorthy, N., D. Tan, M. Ismail, and R. Meier. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behavior of 27 species of sepsid flies (Diptera: Sepsidae). Journal of Evolutionary Biology 22:2146-2156.

Ramos, M., J. A. Coddington, T. E. Christenson, and D. J. Irschick. 2005. Have male and female genitalia coevolved? A phylogenetic analysis of genitalic morphology and sexual size dimorphism in web-building spiders (Araneae : Araneoidea). Evolution 59:1989-1999.

Ritchie, M. G. 2007. Sexual selection and speciation. Annual Review of Ecology Evolution and Systematics 38:79-102.

Schauble, C. S. 2004. Variation in body size and sexual dimorphism across geographical and environmental space in the frogs Limnodynastes tasmaniensis and L-peronii. Biological Journal of the Linnean Society 82:39-56.

Schluter, D., T. D. Price, and L. Rowe. 1991. Conflicting selection pressures and life-history trade-offs. Proceedings of the Royal Society of London Series B-Biological Sciences 246:11-17.

Schulz, K. S. 1999. The evolution of mating systems in black scavenger flies (Diptera: Sepsidae). Doctoral dissertation, Department of Entomology, University of Arizona.

Serrano-Meneses, M. A., A. Cordoba-Aguilar, V. Mendez, S. J. Layen, and T. Szekely. 2007. Sexual size dimorphism in the American rubyspot: male body size predicts male competition and mating success. Animal Behaviour 73:987-997.

Shine, R., and M. Fitzgerald. 1995. Variation in mating systems and sexual size dimorphism between populations of the australian python *Morelia spilota* (Serpentes, Pythonidae). Oecologia 103:490-498.

Stillwell, R. C., W. U. Blanckenhorn, T. Teder, G. Davidowitz, and C. W. Fox. 2010. Sex Differences in Phenotypic Plasticity Affect Variation in Sexual Size Dimorphism in Insects: From Physiology to Evolution. Pp. 227-245. Annual Review of Entomology.

Stillwell, R. C., and C. W. Fox. 2007. Environmental effects on sexual size dimorphism of a seed-feeding beetle. Oecologia 153:273-280.

Storz, J. F., J. Balasingh, H. R. Bhat, P. T. Nathan, D. P. S. Doss, A. A. Prakash, and T. H. Kunz. 2001. Clinal variation in body size and sexual dimorphism in an Indian

fruit bat, Cynopterus sphinx (Chiroptera : Pteropodidae). Biological Journal of the Linnean Society 72:17-31.

Tammaru, T., T. Esperk, V. Ivanov, and T. Teder. 2010. Proximate sources of sexual size dimorphism in insects: locating constraints on larval growth schedules. Evolutionary Ecology 24:161-175.

Tan, D., Y. Ang, G. Lim, M. Ibrahim, and R. Meier. 2010. From 'cryptic species' to integrative taxonomy: an iterative pro sequences, morphology, and behaviour leads to the resurrection of *Sepsis pyrrhosoma* (Sepsidae: Diptera). Zoologica Scripta.

Teder, T., and T. Tammaru. 2005. Sexual size dimorphism within species increases with body size in insects. Oikos 108:321-334.

Teuschl, Y., and W. U. Blanckenhorn. 2007. The reluctant fly: what makes Sepsis cynipsea females willing to copulate? Animal Behaviour 73:85-97.

Teuschl, Y., C. Reim, and W. U. Blanckenhorn. 2007. Correlated responses to artificial body size selection in growth, development, phenotypic plasticity and juvenile viability in yellow dung flies. Journal of Evolutionary Biology 20:87-103.

Teuschl, Y., C. Reim, and W. U. Blanckenhorn. 2010. No size-dependent reproductive costs in male black scavenger flies (Sepsis cynipsea). Behavioral Ecology 21:85-90.

Yu, B.-G., R.-Q. Zheng, Y. Zhang, and C.-T. Liu. 2010. Geographic variation in body size and sexual size dimorphism in the giant spiny frog Paa spinosa (David, 1875) (Anura: Ranoidae). Journal of Natural History 44:1729-1741.

Zerbe, F. 1993. Innerartliche Größenvariabilität und Paarungsverhalten bei *Sepsis punctum* (Fabricius, 1794) [Diptera, Sepsidae]. Diplomarbeit der Fakultät Biologie der Julius-Maximilians- Universität Würzburg.

CHAPTER TWO

Differential investment in pre- versus post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae)

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ABSTRACT

Theory predicts that males have a limited amount of resources to invest in reproduction, suggesting a trade-off between traits that enhance mate acquisition and those enhancing fertilization success. Here we investigate the relationship between pre- and post-copulatory investment by comparing the mating behavior and reproductive morphology of four European and five North American populations of the dung fly *Sepsis punctum* (Diptera) that display a reversal of sexual size dimorphism (SSD). We show that the geographic reversal in SSD between the continents (male-biased in Europe, female-biased in North America) is accompanied by differential investment in pre- versus post-copulatory traits. We find higher re-mating rates in European populations, where larger males acquire more matings and consequently have evolved relatively larger testes and steeper hyper-allometry with body size. American populations, in sharp contrast, display much reduced, if any, effect of body size on those traits. Instead, North American males demonstrate an increased investment in mate acquisition prior to copulation, with more mounting attempts and a distinctive abdominal courtship display that is completely absent in Europe. When controlling for body size, relative female spermathecal size is similar on both continents, so we find no direct evidence for the co-evolution of male and female internal reproductive morphology. By comparing allopatric populations of the same species that apparently have evolved different mating systems and consequently SSD, we thus indirectly demonstrate differential investment in pre- vs. post-copulatory mechanisms increasing reproductive success.

KEYWORDS

Sepsid flies; population differentiation; speciation; testes; spermathecae; mating behavior.

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1. INTRODUCTION

Understanding how sexual selection contributes to phenotypic divergence within and between species has received considerable interest in evolutionary biology. Sexual selection, as originally conceived by Darwin, describes the variation in reproductive success due to differences among individual males in acquiring mates (Darwin, 1871). However, it is now clear that sexual selection often extends far beyond the initiation of copulation. Post-copulatory processes such as cryptic female choice (Eberhard, 1985, 1996) and sperm competition (Parker, 1970; Simmons, 2001b, 2005), as well as sexual conflict over control of fertilization (Parker, 1979; Arnqvist & Rowe; 2005; Parker, 2006) are recognized as important determinants of reproductive success in polyandrous species that mate multiply. Recent years have witnessed increased research across a broad range of taxa on the diversity of male adaptations that serve to enhance a male's fertilization success relative to that of other males (Simmons, 2001a). However, an issue remains as to how pre- and post-copulatory sexual selection interact in shaping the evolution of trait complexes (Arnqvist & Danielsson, 1999; Simmons, 2001b; Markow, 2002; Emlen et al., 2005a).

Males have limited resources to invest in reproduction, which they must allocate to mate acquisition and, given copulations, successful inseminations and fertilizations (Ball & Parker, 1996; Parker et al., 1997; Simmons, 2001a). This suggests a fundamental trade-off between traits that enhance mating success and those that influence fertilization success, the combination of which is expected to vary with the mating system (Simmons & Emlen, 2006; reviewed in Parker & Pizzari, 2010). For instance, in strongly polyandrous groups with female-biased sex ratio, high female re-mating rates could intensify sperm competition but may relax male competition over access to receptive females at breeding sites. The opposite pattern may be expected if females rarely re-mate, implying relaxed sperm competition but perhaps more intense competition among males prior to mating (Reuter et al., 2008). Examples of an allocation trade-off are apparent in the evolutionary diversification of beetle horns (Emlen et al., 2005b; Simmons et al., 2007), the contrasting patterns of courtship display traits and sperm characteristics among lineages of *Drosophila* (Pitnick, 1996; Markow, 2002), or the association between male courtship attractiveness and paternity share in fireflies (Demary & Lewis, 2007).

While certain traits will be predominantly favored by either pre- or post-copulatory sexual selection, other traits, such as body size, are clearly important in both (Holleley et al., 2006). Large body size, as well as the expression of countless and diverse primary and secondary sexual traits, is often favored by classic male-male competition or female

choice (reviewed in Andersson, 1994; Blanckenhorn, 2000; Fairbairn et al., 2007; Hunt et al., 2009). For instance, during aggressive contests for access to females that are typical in many animal species, large males are often more successful at acquiring mates and/or forcing copulations (Parker & Thompson, 1980; Zucker & Murray, 1996; Shine & Mason, 2005; Brown, 2008; Hasegawa et al., 2011; Jorge & Lomonaco, 2011). Large body size can also confer a mating advantage in non-combative courtship displays, as observed in certain insects and anurans (Howard & Young, 1998; Simmons, 1988). The effect of body size on mate acquisition is particularly apparent in species that display size-dependent alternative mating tactics. In a number of fishes and birds (Ryan et al., 1992; Brantley & Bass, 1994; Lank et al., 1995), and even insects such as the rove beetles and yellow dung flies (Forsyth & Alcock, 1990, Pitnick et al., 2009), large and small males can develop completely different pre-copulatory strategies towards attaining matings that might involve sneaking, cuckoldry or even female mimicry (reviewed in Gross, 1996, Schuster & Wade, 2003).

Body size also affects post-copulatory processes via correlated morphology and allometric scaling (Simmons, 2001a). Relative testis size, i.e. sperm production in relation to body size, is commonly used to gauge sperm competitive ability. In general, testis size scales positively, albeit typically hypo-allometrically with body size, both within (Gage et al., 1995; Tomkins & Simmons, 2002; Wedell et al., 2006) and across species (Møller, 1988, 1989; Gage, 1994; Hosken, 1997; Stockley et al., 1997; Schulte-Hostedde & Millar, 2004; Minder et al., 2005; Schulte-Hostedde & Alarie, 2006; Liao et al., 2011; Vahed et al., 2011). Because larger males consequently harbor absolutely more but relatively fewer sperm, they are presumed to transfer or displace larger quantities of sperm in many species, conferring a fertilization advantage (reviewed in Simmons, 2001a; Bangham et al., 2002). In other species, however, there is no size advantage (Stockley & Purvis, 1993; Parker & Simmons, 1994; Arnqvist & Danielsson, 1999, Tomkins & Simmons, 2002) or at times even a small male advantage in sperm competition (Schneider et al., 2000; Danielsson, 2001; Sato et al., 2004; Schneider & Elgar, 2005; Wenninger & Averill, 2006; Watt et al., 2011), such as when smaller males invest disproportionally more in testes or ejaculates (Schulte-Hostedde & Millar, 2004; Schäfer et al., 2008; Schütz et al., 2010).

We here study the relationship between pre- and post-copulatory sexual selection by comparing the mating behavior and reproductive morphology of four European and five North American populations of the black scavenger or dung fly *Sepsis punctum* (Fabricius, 1794) (Diptera: Sepsidae). Sepsid flies are increasingly used as model organisms in sexual selection studies because they have diverse sexual dimorphisms as

well as elaborate mating behavior (Puniamoorthy et al., 2008, 2009) and can be reared easily in the laboratory (e.g. Teuschl & Blanckenhorn, 2007). *Sepsis punctum* is a geographically widespread species that can be collected not only on cattle pastures but also on dog excrements in parks and open fields (Pont & Meier, 2002). In a recent study (Puniamoorthy et al., 2012), we found that the intensity of pre-copulatory sexual selection acting on male body size was much stronger in European than in North American populations. In agreement with the differential equilibrium hypothesis of SSD (Blanckenhorn, 2000; Fairbairn et al., 2007), this can explain the geographic reversal in sexual size dimorphism (SSD) of *S. punctum* observed between the continents. Schulz (1999) first noticed that the presence of pre-copulatory courtship also varies between the continents, suggesting that the reversal in SSD might segregate with differences in the mating system and other trait complexes. We here explore this further by focusing on traits related to mate acquisition and traits with putative function in sperm competition. We took an integrated approach, comparing detailed behavioral experiments and observations with morphological measures of fertilization-related structures and body size across nine cross-continental *S. punctum* populations.

2. METHODS

2.1. Sampling of populations

We collected flies from four European sites, Nyköping, Sweden (58.67°N, 16.94°E), Berlin, Germany (52.45°N, 13.28°E), Vienna, Austria (48.20°N, 16.36°E), Zürich, Switzerland (47.40°N, 8.55°E) as well as five North American populations from Davis, California (38.54°N, -121.75°W), Park City, Utah (40.66°N, -111.52°E), Athens, Georgia (33.96°N, -83.38°E), Manhattan, New York (40.78°N, -73.96°E) and Ottawa, Ontario (45.42°N, -75.67°E). We caught gravid females on and around fresh dung pats in open cow pastures, transported them back to the laboratory in Zurich, and used them to establish stock cultures of 10-20 iso-female lines per population. Alternatively, we set out small pots of cow dung in city parks overnight for a few days and shipped them back to the laboratory. The emergent flies from each pot were treated as single lines. All fly cultures were housed in separate clear plastic containers, reared in a climate chamber at standardized 24°C, 60% humidity, 14 h light cycle, and were regularly supplied with fresh cow dung, sugar and water *ad libitum*.

2.2. Rearing of flies for experiments

In order to generate a range of phenotypic body sizes, we provided stock cultures of each population pots with different amounts of cow dung and allowed for oviposition overnight. We transferred these pots into another container and reared them under the above-mentioned standard conditions. After approximately two weeks of juvenile

development, we sexed emerging flies individually under a microscope within 24 hours of eclosion, and subsequently housed virgin males and females in separate containers.

2.3. Morphological study

For each population, we randomly selected approximately 50 to 140 individuals from the 'virgin' containers and froze them at -20 ^{o}C overnight. We then measured the flies for body size (head width) before dissecting them under the microscope in Ringer's solution under a Leica MS 5 microscope. We transferred both male testes and both female spermathecae on a concave glass slide with a drop of Ringer's and cover slide. We calculated the volume of the respective reproductive structures from the measurements of the length and width of both testes (ellipsoid) and the diameter of both spermathecae (sphere) under a Zeiss light microscope. We had assistance from several students (blocking factor in the statistical analysis).

2.4. Behavioral study

2.4.1. Mating trials with virgin flies

We conducted all mating trials 3-4 days after eclosion to ensure sexual maturity (cf. Teuschl & Blanckenhorn 2007; Puniamoorthy et al., 2012). We randomly selected a male and a female from the 'virgin' containers and introduced them into a clear glass vial (containing cow dung smeared on a small filter paper) to observe their interaction for a maximum of one hour or until copulation occurred. We recorded the number of male mounting attempts, the number of courtship displays, the latency in time to copulation, as well as the copulation duration (cf. Ding & Blanckenhorn, 2002). We conducted these mating trials until we reached our targeted sample size of ca. 20 mated pairs per population.

2.4.2. Re-mating trials with mated individuals

Upon successful copulation, we separated the mated pair, housed each male and female in a new glass vial (with dung, sugar and water) and gave them individual identification labels. One week after the first copulation, we conducted re-mating trials, randomly assigning each male to a new female, and again recorded all interactions for a maximum of one hour in a new glass chamber. At the end of the trial, we returned each fly to its individual 'home' vial (replenished with fresh dung, sugar and water). We repeated these re-mating trials for both sexes for a maximum of eight weeks or until the flies died, at which point they were frozen and measured for body size.

2.5. Statistical analyses

In order to quantify differential allocation in male pre-copulatory courtship display, we created an index by summing both the number of male mounting attempts and the number of courtship displays (both counts), correcting for the duration of each trial (via residuals). We scored copula duration, mating and re-mating frequencies as separate dependent variables. For the reproductive structures, we took the mean (of two) testes volume and mean spermathecal volume.

We performed significance testing using ANCOVA with continent as fixed, population nested within continent as random factor, and body size as a continuous covariate (unless otherwise mentioned). Body size was z-score standardized before analysis in all ANCOVAs such that all factors, and especially the main effects, are properly evaluated at the center of the actual data distribution. For overall body size, copulation duration and re-mating frequencies, sex was included as an additional factor. All volume measurements were cube root transformed to the linear scale. We initially included all relevant interaction terms, which were dropped from the model if not significant, except when required as error terms in testing higher level effects (for details see Supplementary file 1). We conducted all analyses using the software IBM SPSS Statistics version 19.0 (SPSS, Inc.).

3. RESULTS

3.1. Scaling relationships between traits

As expected by our earlier study (Puniamoorthy et al. 2012), we found sexual size dimorphism to be female-biased in North America and male-biased in Europe (continent by sex interaction for body size: $F_{1,7}$= 20.23, P = 0.002; Table 1). Populations within continents also varied with respect to overall body size ($F_{7,879}$= 6.71, P = 0.008), and there was additional (but uninteresting) variation introduced by measurers (blocking effect: $F_{18,879}$= 3.91, P < 0.001).

Female spermathecal size was strongly positively (but hypo-allometrically) related to body size (Figure 1A; $F_{1,439}$ = 141.07, P < 0.001; overall regression equations based on log-transformed linear measures (± 95% CI): [Europe] y= -1.281 (± 0.069) + 0.437x (± 0.116), [N. America] y= -1.389 (± 0.077) + 0.585x (± 0.131)). The average spermathecal volume ranged from 0.173 ± 0.056 SE (x 10^{-3} mm^3) in the California population to 0.254 ± 0.092 SE (x 10^{-3} mm^3) in the German population (Table 1). There was variation due to measurer and between populations (blocking effect: $F_{17,439}$ = 5.76, P < 0.001; population effect: $F_{7,439}$ = 9.27, P < 0.001). However, relative (i.e. size-controlled) spermathecal size did not vary significantly among continents (continent

effect: $F_{1,7}$ = 0.876, P = 0.378). Furthermore, the allometric relationship between spermathecae and body size was the same for all populations and on both continents (i.e. population by body size and continent by body size interactions were n.s.; for details see Appendix A).

Overall, we found that larger males had bigger testes (strong main effect of body size: $F_{1,378}$ = 623.27, P < 0.001). The average testes volume varied drastically among populations up to 5-fold, ranging from 0.149 ± 0.046 SE (x 10^{-2} mm^3) in New York to 0.718 ± 0.469 SE (x 10^{-2} mm^3) in Austria. Even after controlling for body size, both the relative testes size and the testes-body size allometry within continents were significantly different (population main effect: $F_{7,378}$ = 7.86, P < 0.001; population by body size interaction: $F_{7,378}$ = 10.13, P < 0.001). Crucially, there was an especially strong systematic difference between the continents (continent main effect: $F_{1,7}$ = 102.83, P < 0.001), with the populations in Europe having larger testes and testes displaying a much steeper hyper-allometric relationship with body size (Figure 1B; body size by continent interaction: $F_{1,7}$ = 24.77, P = 0.002 ; regression equations based on log-transformed linearized measures (± 95% CI): [Europe] y = -1.285 (± 0.069) + 1.389x (± 0.114), [N. America] y = -0.911 (± 0.102) + 0.551x (± 0.174)).

3.2. Mounting attempts

The number of male mounting attempts until successful copulation differed significantly between the continents (Table 2). Many copulations in Europe were attained by the first male mounting attempt, whilst American males had to work much harder to gain a successful mating, ranging from 2.70 ± 2.32 SE attempts in California to 5.14 ± 2.19 SE attempts in Ottawa (Figure 2A; continent main effect: $F_{1,\ 7}$ = 12.56, P = 0.009; population main effect: $F_{7,\ 163}$ = 7.32, P < 0.001; body size effect and all body size by factor interactions n.s.).

3.3. Pre-copulatory courtship display and intensity

One major difference between the two continents is the absence of an abdominal courtship display in all four European populations (Table 2; see Appendix B). Additionally, the intensity of displays varied strongly among the American populations, with relatively low occurrence in the California and Park City populations. Consequently we found significant variation between the continents as well as among the populations for the combined index of investment in mate acquisition (subsuming mounting and courtship attempts), the latter being driven mainly by the variation between the courting American populations (Figure 2B; continent effect: $F_{1,\ 7}$ = 9.98, P = 0.039; population

effect: $F_{7, 163}$ = 30.84, P < 0.001; body size main effect and all body size by factor interactions n.s.).

3.4. Female mating rates

The mating rate of virgin flies varied strongly between populations (matings/total trials: Austria=27/50; Germany=25/49; Switzerland=24/70; Sweden=23/57; California=20/93; Georgia=18/109; New York=22/88; Ottawa=7/170; Park City=12/130), with the European populations mating more readily than the American populations (binary logistic model on 1/0 data; continent effect: $\chi^2_{1,7}$ = 66.50, P < 0.001; population effect: $\chi^2_{7, 780}$ = 38.16, P < 0.001).

3.5. Male and female re-mating behavior

Due to the extremely low mating rates in the Ottawa and Park City populations, we did not include them in the re-mating study. For the remaining seven populations, we conducted weekly mating trials and found that the number of copulations over 6-8 weeks varied between the continents (Table 2; continent main effect: $F_{1,280}$ = 27.02, P = 0.003). Interestingly, larger females tended to re-mate less frequently, whilst larger males attained more copulations (Figure 3B). This effect was exclusively driven by the European populations (sex by body size interaction: $F_{1, 280}$ = 12.53, P < 0.001; three-way sex by continent by body size interaction: $F_{2, 10}$ = 6.67, P = 0.014; body size main effect and all other factor by body size interactions n.s.)

3.6. Copulation duration

Copulation duration in *S. punctum* typically varied from 20 to 30 minutes (Table 2), strongly depending on body size such that larger females and smaller males copulated for longer (Figure 3A; male body size effect: $F_{1, 162}$ = 43.93, P < 0.001; female body size effect: $F_{1, 162}$ = 10.08, P = 0.002). Despite differences between populations, the continental origin of the flies did not significantly influence copulation duration (continent main effect: $F_{1, 7}$ = 1.95, P = 0.205; population main effect: $F_{7, 162}$ = 3.32, P = 0.002; all corresponding factor by body size interactions n.s.)

Figure 1A. Cube-root transformed (linearized) spermathecae volume against female body size.

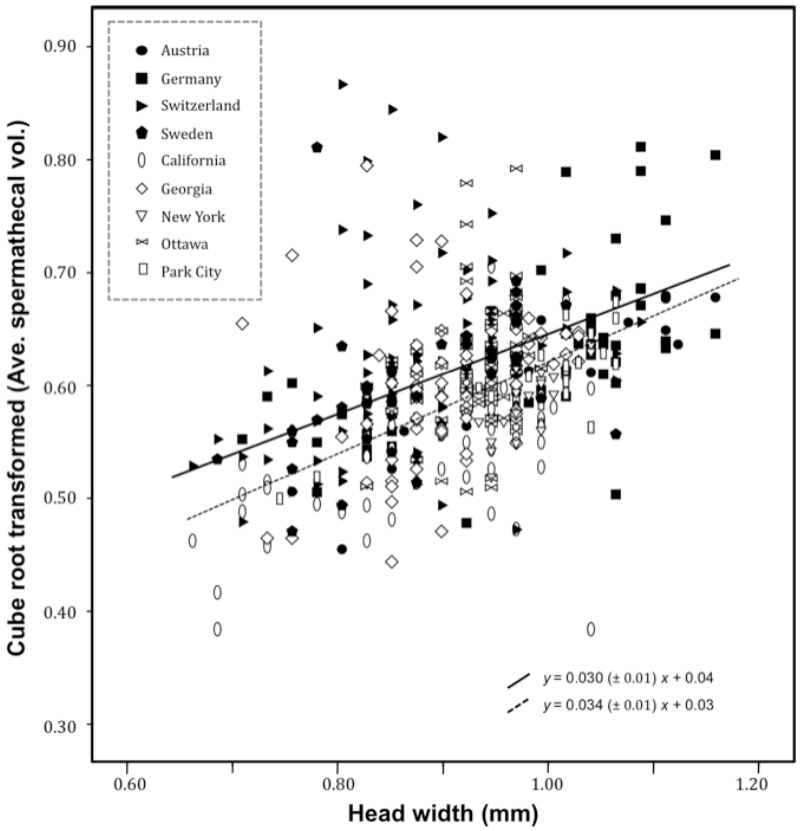


Figure 1B. Cube-root transformed (linearized) testis volume against male body size.

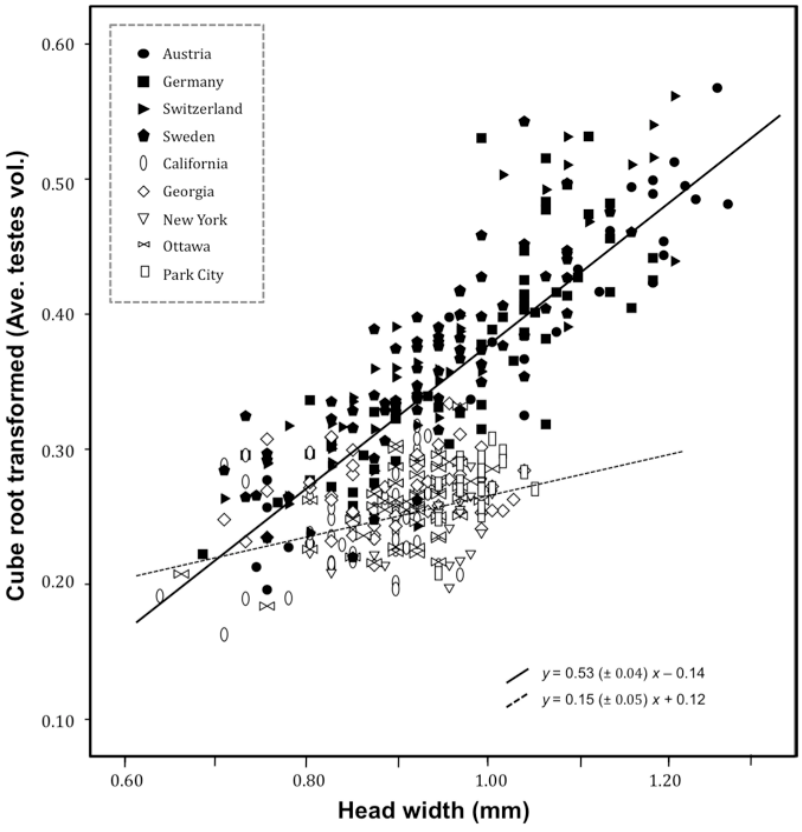


Figure 2A. Average (± SE) mounting attempts

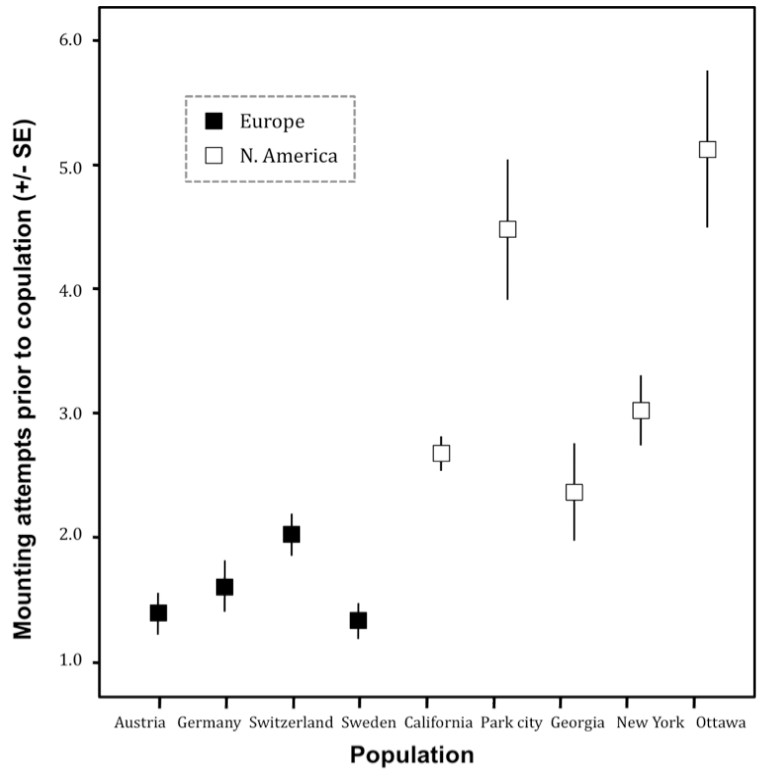


Figure 2B. Average (± SE) pre-copulatory investment, i.e. combined mounting and courtship displays. Dashed circle highlights American west-coast populations.

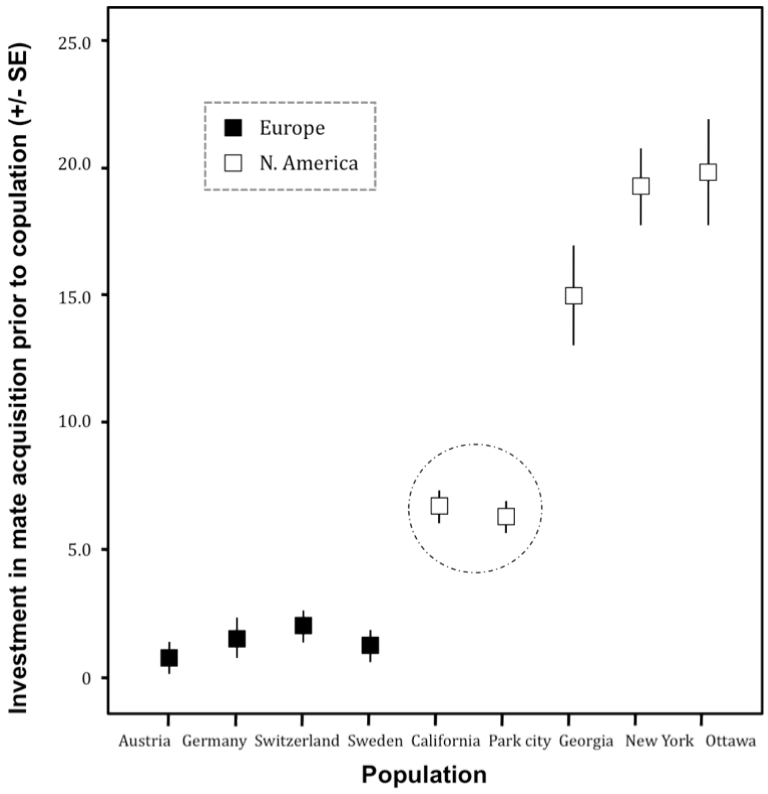


Figure 3A. Copula duration of females and males in 9 populations

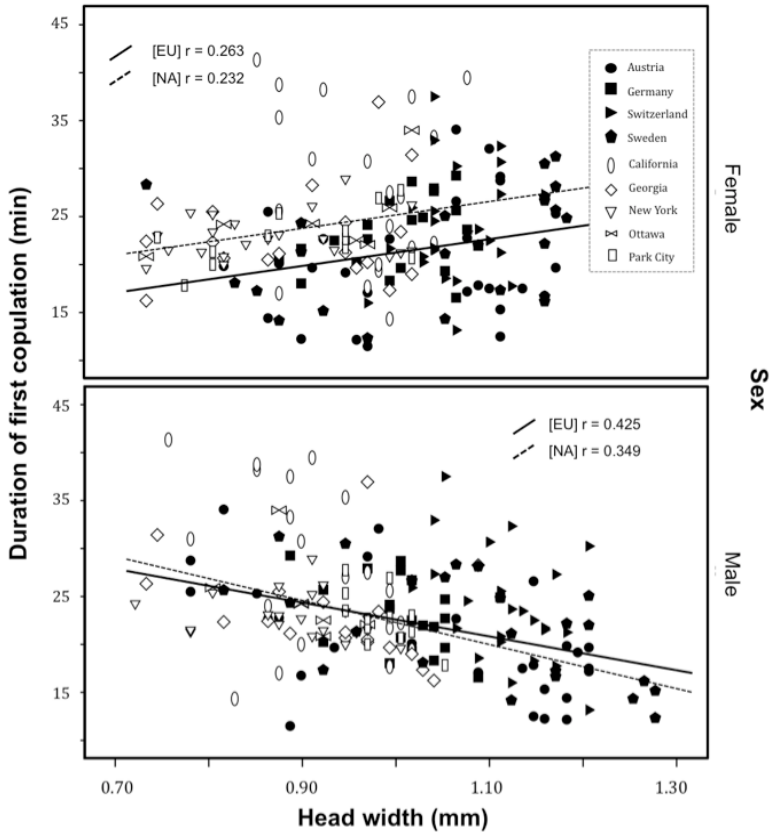


Figure 3B. Re-mating frequency in 7 populations as a function of body size.

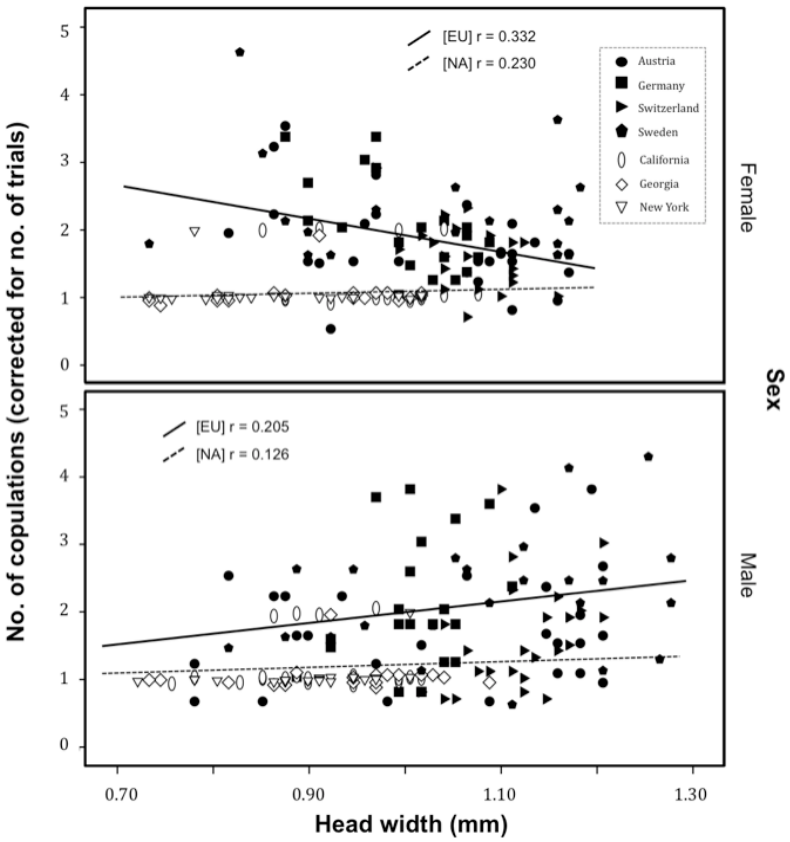


Table 1. Population means (± SE) for morphology data: body size, average male testes volume, and average female spermathecal diameter and volume [n, sample size].

Population	Female				Male		
	Ave. spermathecal diameter (x10 mm)	Ave. spermathecal volume (x 10^{-3} mm^3)	Head width (mm)	n	Ave. testes volume (x 10^{-2} mm3)	Head width (mm)	n
Austria	0.919 ± 0.095	0.220 ± 0.062	0.976 ± 0.109	23	0.718 ± 0.469	1.043 ± 0.180	27
Germany	0.959 ± 0.100	0.254 ± 0.092	0.970 ± 0.119	59	0.568 ± 0.350	0.977 ± 0.121	59
Switzerland	0.958 ± 0.123	0.250 ± 0.103	0.873 ± 0.084	88	0.569 ± 0.443	0.934 ± 0.131	47
Sweden	0.930 ± 0.099	0.228 ± 0.076	0.884 ± 0.089	37	0.523± 0.280	0.938 ± 0.100	73
California	0.845 ± 0.094	0.173 ± 0.056	0.833 ± 0.105	67	0.163 ± 0.066	0.871 ± 0.076	57
Georgia	0.924 ± 0.100	0.224 ± 0.072	0.907 ± 0.068	73	0.200 ± 0.061	0.898 ± 0.081	52
New York	0.906 ± 0.044	0.206 ± 0.029	0.986 ± 0.030	25	0.149 ± 0.046	0.947 ± 0.054	21
Ottawa	0.947 ± 0.082	0.237 ± 0.067	0.929 ± 0.040	75	0.181 ± 0.056	0.912 ± 0.057	57
Park city	0.937 ± 0.072	0.227 ± 0.047	0.890 ± 0.088	19	0.214 ± 0.045	0.990 ± 0.028	21

Table 2. Population means (± SE) for body size and pre- and post-copulatory behavior data ['/' means no data available; n, sample size].

Population			Investment in mate acquisition			Investment in post-copulatory selection			
	Male head width (mm)	n	Ave. no of mounts	Ave. no of courtship displays	Ave. precop. investment (corrected for duration)	Ave. no of re-mating trials	Ave. no of copulations	Ave. duration of first copulation (min)	Ave. re-mating frequency (corrected for number of trials)
Austria	1.04 ± 0.15	27	1.41 ± 0.97	0	1.41 ± 0.91	5.67 ± 2.86	1.78 ± 0.93	20.37 ± 6.11	1.77 ± 0.91
Germany	1.01 ± 0.05	21	1.62 ± 1.40	0	1.62 ± 0.98	6.62 ± 1.50	2.14 ± 1.06	23.11 ± 3.71	1.96 ± 0.98
Switzerland	1.12 ± 0.05	24	2.04 ± 1.04	0	2.04 ± 0.86	5.17 ± 2.53	1.62 ± 0.82	23.96 ± 5.82	1.63 ± 0.65
Sweden	1.09 ± 0.14	23	1.35 ± 0.71	0	1.35 ± 0.65	6.74 ± 2.01	2.22 ± 0.90	21.74 ± 5.64	2.22 ± 0.81
California	0.91 ± 0.07	20	2.70 ± 2.32	1.20 ± 1.85	3.90 ± 2.12	6.10 ± 2.20	1.20 ± 0.41	28.11 ± 8.48	1.19 ± 0.40
Georgia	0.93 ± 0.10	18	2.39 ± 2.00	12.61 ± 14.28	15.00 ± 8.51	4.17 ± 1.76	1.06 ± 0.24	23.17 ± 5.11	1.06 ± 0.22
New York	0.87 ± 0.10	21	3.05 ± 2.00	16.23 ± 15.51	19.27 ± 7.15	4.86 ± 2.68	1.05 ± 0.21	22.92 ± 2.36	1.05 ± 0.22
Ottawa	0.90 ± 0.05	7	5.14 ± 2.19	14.71 ± 11.73	19.86 ± 5.48	/	/	24.84 ± 4.37	/
Park city	0.89 ± 0.09	12	4.50 ± 4.25	0.83 ± 1.40	5.33 ± 2.01	/	/	22.80 ± 2.84	/

4. DISCUSSION

We show that the geographic reversal in SSD between European and North American populations of the dung fly *Sepsis punctum* is accompanied by differential allocation in traits engaged in pre- versus post-copulatory sexual selection. European populations display a higher mating propensity and males evolved relatively larger testes and much steeper, positive or hyper-allometry with body size, in accordance with sperm competition theory predicting higher investment in sperm production with increasing level of sperm competition (Parker et al., 1997; Parker and Pizzari, 2011). In sharp contrast, North American populations show a much lower female mating rate, and males invest more in mate acquisition prior to copulation. Their mating system is characterized by more male mounting attempts and by the presence of a distinctive abdominal courtship display, which is completely absent in Europe. At the same time, we found the intensity of pre-copulatory sexual selection on male body size, in terms of the cumulative number of matings over a significant portion of the lifetime (6-8 weeks), to be much stronger in European populations displaying male-biased SSD than in North American populations with female-biased SSD (Figure 3B), corroborating previous findings based on single mating probabilities (Puniamoorthy et al., 2012). By comparing allopatric populations of the same species that apparently have evolved different mating systems and consequently SSD, we thus demonstrate differential allocation of European and North American flies in pre- vs. post-copulatory mechanisms affecting reproductive success (Markow, 2002), analogous to comparisons among intra-specific morphs with alternative mating strategies (Gage et al., 1995; Tomkins & Simmons, 2002; Kelly, 2008).

Theory predicts an optimal mating rate for females beyond which multiple matings can have detrimental effects (Arnqvist, 1989; Firman & Simmons, 2008; Simmons & Garcia-Gonzalez, 2008), as copulation can increase predation risk, decrease foraging ability (Daly, 1978; Sih et al., 1990; Fairbairn, 1993), or even produce internal injury due to male genital structures (Crudgington & Siva-Jothy, 2000; Blanckenhorn et al., 2002). Given that males usually profit from multiple matings and females often do so to a much lesser extent, this can potentially generate conflict over mating and fertilization (Bateman, 1948; Arnqvist et al., 2000, Arnqvist & Rowe, 2005). Studies show that in species with male-biased SSD, male resource defense polygyny, territoriality, and monopolization of females are common, whereas in species with female-biased (or no) SSD, female choice and male courtship displays dominate (Andersson, 1994; Ding & Blanckenhorn, 2002; Arnqvist & Rowe, 2005). This predicts that external or internal courtship facilitating female choice should be more apparent, or should more likely evolve, in species (or populations) with female-biased SSD (Eberhard, 1996), as is the

case for North American but not European *S. punctum,* the latter displaying male-biased SSD.

Moreover, according to theory (Parker et al., 1997), female re-mating rate should positively correlate with relative testis size both within (e.g. Gage, 1994; Gage et al., 1995; Wedell, 1997; Tomkins & Simmons, 2002; this study) and among species (e.g. Møller, 1988, 1989; Gage, 1994; Hosken, 1997; Stockley et al., 1997; Minder et al., 2005). Thus European *S. punctum* males, which face greater risk of sperm competition because of higher female (re-)mating rates, evolved relatively larger testes and a steep testes/body size hyper-allometry as compared to North American populations of this species, presumably in connection with the change in mating system (Figure 1B). Although it could in principle relate to different sperm competition mechanisms, this difference in allometry most parsimoniously reflects the necessity of producing more sperm to increase fertilization chances in a fair or loaded raffle (Parker, 1970; Short, 1979; Simmons, 2001a).

Copulation duration and male investment in transferred ejaculates can depend on various factors such as the risk of sperm competition and/or the quality of the females (reviewed in Kelly and Jennions, 2011). In fact, a recent study of the closely related *Sepsis cynipsea* documented that males invest more sperm in more fecund females with smaller males copulating longer and copulations lasting longer with larger females (Teuschl et al., 2010; cf. Lefranc & Bundgaard, 2000, for *Drosophila*). Our data (Figure 3A) similarly indicate that, equally on both continents, copulation duration in *S. punctum* increases with female size and decreases with male size, presumably because, physiologically, larger females require and can store more sperm because they produce more eggs, and probably larger males have wider ducts and can transfer more sperm quickly (Simmons, 2001b; Blanckenhorn et al., 2004). An additional or alternative explanation could be a potential trade-off between polyandry and relative ejaculate expenditure (Vahed & Parker, 2012). For instance, given their increased mating rate, larger males might copulate for shorter time because they invest less sperm per copulation, and, conversely, small males invest relatively more per copulation because of their reduced future mating probability (Parker & Ball, 2005; Fromhage et al., 2008; Vahed et al., 2011). So far, little is known about the detailed mechanisms of sperm competition in any sepsid species. Based on behavioral observations and data on male sperm investment, Martin and Hosken (2002) concluded a 'raffle' mechanism of sperm competition for the related *S. cynipsea*. However, preliminary paternity analysis in *S. punctum* based on twelve doubly mated females of a German population indicate almost complete last male sperm precedence (Schulz 1999), so sperm competition mechanisms

might be quite variable among *Sepsis* species. Clearly, the relationship between ejaculate allocation and relative paternity success in sepsid flies requires further study.

Sexual selection has been considered as an important barrier to gene flow through its direct effects on mate or gamete recognition (Panhuis et al., 2001; Servedio & Noor, 2003; Coyne & Orr, 2004). Several comparative studies have documented co-evolution between traits engaged in insemination and fertilization (including testis size and female reproductive tract morphology) (Presgraves et al., 1999; Brown & Eady, 2001; Minder et al., 2005; Rugman-Jones & Eady, 2008; Rönn et al., 2011; Thüler et al., 2011), but this appears not to be the case in *S. punctum*. In contrast to testes size, which was highly differentiated between American and European populations, the number, relative size and allometry of the female spermathecae (the sperm storing organs) is highly conserved across the continents and populations, consistent with a recent comparative study of the internal female reproductive tract of 41 sepsid species (Puniamoorthy et al., 2010). Said study additionally documents some morphological variation in a secondary sperm storage organ, the ventral receptacle. In other acalyptrate Diptera, such as the Mediterranean fruit fly, the ventral receptacle is the first sperm storage organ to deplete, suggesting that these delicate structures could be the likely site of fertilization (De Carlo et al., 1994). However, we did not treat this in our study because in *S. punctum* this unsclerotized, membranous structure, which is very much smaller than the spermathecae (approx. < 0.1 mm), is difficult to visualize without staining and even harder to dissect without destroying it.

We document that male courtship behavior varied significantly both between and within continents. In particular, our study suggests that among the North American populations, there could be a strong east-west gradient in the intensity of male display (Figure 2B). Furthermore preliminary data based on neutral molecular markers indicate genetic differentiation within American populations of *S. punctum* (N. Puniamoorthy, unpublished data). This scenario is consistent with mountain ranges and glaciation events in America that have been documented to limit gene flow and result in population divergence and speciation (e.g. Hewitt 2004, Mirol et al., 2007). Further work on variation in courtship in North America is needed to investigate the significance of the abdominal display in possibly establishing pre-copulatory barriers to gene flow.

In summary, we demonstrate a shift in mating system and associated changes in behavior and morphology when comparing cross-continental populations of the widespread dung fly *S. punctum*. North American populations of this species are smaller, display female-biased SSD, low (re-)mating rates, high investment in pre-copulatory

courtship and mating attempts, and hypo-allometric testes/body size scaling, whereas European populations are larger in size, display male-biased SSD, higher (re-)mating rates, no pre-copulatory courtship, and steeper hyper-allometric testes/body size scaling (Figure 1, 2). Because the allometry of female spermatheca size to body size is similar on both continents, we have no indication of co-evolution of male and female internal reproductive morphology by sexual selection or conflict (cf. Thüler et al., 2011). Our results imply and demonstrate, across populations of a single species, differential investment in pre- vs. post-copulatory traits indicative of a putative trade-off (Parker et al., 1997; Markow, 2002). Which mating system or SSD is the original state, and which sequence of events lead to the divergent evolution of North American and European *S. punctum*, remains to be answered by further studies.

5. REFERENCES

Andersson, M. 1994. *Sexual selection*. Princeton University Press, Princeton, New Jersey.

Arnqvist, G. 1989. Multiple Mating in a Water Strider Mutual Benefits or Intersexual Conflict. *Ani. Behav.***38**: 749-756.

Arnqvist, G. & Danielsson, I. 1999. Copulatory behavior, genital morphology, and male fertilization success in water striders. *Evolution* **53**: 147-156.

Arnqvist, G., Edvardsson, M., Friberg, U. & Nilsson, T. 2000. Sexual conflict promotes speciation in insects. *PNAS* **97**: 10460-10464.

Arnqvist, G. & Rowe, L. 2005. *Sexual Conflict*. Princeton University Press, Princeton.

Ball, M. A. & Parker, G. A. 1996. Sperm competition games: External fertilization and ''adaptive'' infertility. *J Theor. Biol.***180**: 141-150.

Bangham, J., Chapman, T. & Partridge, L. 2002. Effects of body size, accessory gland and testis size on pre- and postcopulatory success in Drosophila melanogaster. *Ani. Behav.***64**: 915-921.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**: 349-368.

Blanckenhorn, W. U. 2000. The evolution of body size: What keeps organisms small? *Quarterly Review of Biology* **75**: 385-407.

Blanckenhorn, W. U., Hellriegel, B., Hosken, D. J., Jann, P., Altwegg, R. & Ward, P. I. 2004. Does testis size track expected mating success in yellow dung flies? *Funct. Ecol.* **18**: 414-418.

Blanckenhorn, W. U., Hosken, D. J., Martin, O. Y., Reim, C., Teuschl, Y. & Ward, P. I. 2002. The costs of copulating in the dung fly Sepsis cynipsea. *Behav. Ecol.* **13**: 353-358.

Brantley, R. K. & Bass, A. H. 1994. Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish *Porichthysnotatus girard* (Teleostei, Batrachoididae). *Ethology* **96**: 213-232.

Brown, D. V. & Eady, P. E. 2001. Functional incompatibility between the fertilization systems of two allopatric populations of Callosobruchus maculatus (Coleoptera : Bruchidae). *Evolution* **55**: 2257-2262.

Brown, W. D. 2008. Size-biased mating in both sexes of the black-horned tree cricket, Oecanthus nigricornis Walker (Orthoptera : Gryllidae : Oecanthinae). *J Ins. Behav.* **21**: 130-142.

Caracristi, G. & Schlotterer, C. 2003. Genetic differentiation between American and European Drosophila melanogaster populations could be attributed to admixture of African alleles. *Molecular Biology and Evolution* **20**: 792-799.

Coyne, J. A. & Orr, H. A. 2004. *Speciation*. Sinauer Associates, Sunderland, MA.

Crudgington, H. S. & Siva-Jothy, M. T. 2000. Genital damage, kicking and early death. *Nature* **407**: 855-856.

Daly, M. 1978. Cost of Mating. *Am. Nat.* **112**: 771-774.

Danielsson, I. 2001. Antagonistic pre- and post-copulatory sexual selection on male body size in a water strider (Gerris lacustris). *Proc. R. Soc. B.* **268**: 77-81.

Darwin, C. 1871. *The Descent of Man, and Selection in Relation to Sex.* John Murray, London.

De Carlo, J.M., Pellerano, G.N. & Martinez, L.I.1994. Saco del oviducto medio de Ceratitis capitata Wied (Diptera: Tephritidae): consideraciones histo- funcionales. *Physis* **49**:19-25.

Demary, K. C. & Lewis, S. M. 2007. Male courtship attractiveness and paternity success in Photinus greeni fireflies. *Evolution* **61**: 431-439.

Ding, A. & Blanckenhorn, W. U. 2002. The effect of sexual size dimorphism on mating behaviour in two dung flies with contrasting dimorphism. *Evol. Ecol. Res.* **4**: 259-273.

Eberhard, W. G. (1985) Sexual Selection and Animal Genitalia. In: *Eberhard, W. G. Sexual Selection and Animal Genitalia. Xii+244p. Harvard University Press: Cambridge, Mass., USA; London, England. Illus.* pp.

Eberhard, W. G. 1996. *Female control: Sexual selection by cryptic female choice*. Princeton University Press, Princeton.

Emlen, D. J., Hunt, J. & Simmons, L. W. 2005a. Evolution of sexual dimorphism and male dimorphism in the expression of beetle horns: Phylogenetic evidence for modularity, evolutionary lability, and constraint. *Am. Nat.* **166**: S42-S68.

Emlen, D. J., Marangelo, J., Ball, B. & Cunningham, C. W. 2005b. Diversity in the weapons of sexual selection: Horn evolution in the beetle genus Onthophagus (Coleoptera : Scarabaeidae). *Evolution* **59**: 1060-1084.

Fairbairn, D. J. 1993. Costs of loading associated with mate-carrying in the waterstrider, *Aquarius remigis. Behav. Ecol.* **4**: 224-231.

Fairbairn, D. J., Blanckenhorn, W. U. & Szekely, T. 2007. *Sex, size and gender roles: evolutionary studies of sexual size dimorphism.* Oxford University Press, London, UK.

Firman, R. C. & Simmons, L. W. 2008. The frequency of multiple paternity predicts variation in testes size among island populations of house mice. *J. Evol. Biol.* **21**: 1524-1533.

Forsyth, A. & Alcock, J. 1990. Ambushing and prey-luring as alternative foraging tactics of the fly-catching rove beetle *Leistotrophus versicolor* (Coleoptera: Staphylinidae). *J. Ins. Beh.* **3**: 703-718.

Fromhage, L., McNamara, J. M. & Houston, A. I. 2008. Sperm allocation strategies and female resistance: A unifying perspective. *Am. Nat.* **172**: 25-33.

Gage, M. J. G. 1994. Associations between body size, mating pattern, testis size and sperm lengths across butterflies. *Proc. R. Soc. B.* **258**: 247-254.

Gage, M. J. G., Stockley, P. & Parker, G. A. 1995. Effects of alternative male mating strategies on characteristics of sperm production in the Atlantic salmon (Salmo salar): Theoretical and empirical investigations. *Phil. Trans. R. Soc. B* **350**: 391-399.

Gross, M. R. 1996. Alternative reproductive strategies and tactics: Diversity within sexes. *TREE* **11**: 92-98.

Hasegawa, A., Soma, M. & Hasegawa, T. 2011. Male traits and female choice in Java Sparrows: preference for large body size. *Ornithol. Sci.* **10**: 73-80.

Hewitt G. M. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Phil. Trans. R. Soc. Lond. B* **359**: 183–195.

Holleley, C. E., Dickman, C. R., Crowther, M. S. & Oldroyd, B. P. 2006. Size breeds success: multiple paternity, multivariate selection and male semelparity in a small marsupial, Antechinus stuartii. *Mol. Ecol.* **15**: 3439-3448.

Hosken, D. J. 1997. Sperm competition in bats. *Proc. R. Soc. B.* **264**: 385-392.

Howard, R. D. & Young, J. R. 1998. Individual variation in male vocal traits and female mating preferences in Bufo americanus. *Ani. Behav.***55**: 1165-1179.

Hunt, J., Breuker, C. J., Sadowski, J. A. & Moore, A. J. 2009. Male-male competition, female mate choice and their interaction: determining total sexual selection. *J. Evol. Biol.* **22**: 13-26.

Jorge, A. S. & Lomonaco, C. 2011. Body Size, Symmetry and Courtship Behavior of Dysdercus maurus Distant (Hemiptera: Pyrrhocoridae). *Neotrop.l Ento.* **40**: 305-311.

Kelly, C. D. 2008. Sperm investment in relation to weapon size in a male trimorphic insect? *Behav. Ecol.* **19**: 1018-1024.

Kelly, C. D. & Jennions, M. D. 2011. Sexual selection and sperm quality: meta-analyses of strategic ejaculation. *Biol. Rev.* **86**: 863-884.

Lank, D. B., Smith, C. M., Hanotte, O., Burke, T. & Cooke, F. 1995. Genetic polymorphism for alternative mating behavior in lekking male ruff *Philonachus pugnax. Nature* **378**: 59-62.

Lefranc, A. & Bundgaard, J. 2000. The influence of male and female body size on copulation duration and fecundity in Drosophila melanogaster. *Hereditas* **132**: 243-247.

Liao, W. B., Mi, Z. P., Zhou, C. Q., Jin, L., Lou, S. L., Han, X. & Ma, J. 2011. Relative testis size and mating systems in anurans: large testis in multiple-male mating in foam-nesting frogs. *Ani. Biol.* **61**: 225-238.

Markow, T. A. 2002. Perspective: Female remating, operational sex ratio, and the arena of sexual selection in Drosophila species. *Evolution* **56**: 1725-1734.

Martin, O. Y. & Hosken, D. J. 2002. Strategic ejaculation in the common dung fly Sepsis cynipsea. *Ani. Behav.***63**: 541-546.

Minder, A. M., Hosken, D. J. & Ward, P. I. 2005. Co-evolution of male and female reproductive characters across the Scathophagidae (Diptera). *J. Evol. Biol.* **18**: 60-69.

Mirol, P. M., Schaefer, M. A., Orsini, L., Routtu, J., Schloetterer, C., Hoikkala, A. & Butlin, R. K. 2007. Phylogeographic patterns in Drosophila montana. *Mol. Ecol.* **16**: 1085-1097.

Møller, A. P. 1988. Testes size, ejaculate quality and sperm competition in birds. *Biol. J. Linn. Soc.* **33**: 273-283.

Møller, A. P. 1989. Ejaculate quality, testes size and sperm production in mammals. *Funct. Ecol.* **3**: 91-96.

Panhuis, T. M., Butlin, R., Zuk, M. & Tregenza, T. 2001. Sexual selection and speciation. *TREE* **16**: 364-371.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. *Biol. Rev. Cam. Phil. Soc.* **45**: 525-&.

Parker, G. A. (1979) Sexual selection and sexual conflict. In: *Sexual Selection and Reproductive Competition in Insects*, (Blum, M. S. & Blum, N. A., eds.). pp. 123-166. Academic, New Jersey.

Parker, G. A. 2006. Sexual conflict over mating and fertilization: an overview. *Phil. Trans. R. Soc. B* **361**: 235-259.

Parker, G. A. & Ball, M. A. 2005. Sperm competition, mating rate and the evolution of testis and ejaculate sizes: a population model. *Biol. Lett.***1**: 235-238.

Parker, G. A., Ball, M. A., Stockley, P. & Gage, M. J. G. 1997. Sperm competition games: a prospective analysis of risk assessment. *Proc. R. Soc. B.* **264**: 1793-1802.

Parker, G. A. & Pizzari, T. 2010. Sperm competition and ejaculate economics. *Biol. Rev.* **85**: 897-934.

Parker, G. A. & Simmons, L. W. 1994. Evolution of Phenotypic optima and copula duration in dung flies. *Nature* **370**: 53-56.

Parker, G. A. & Thompson, E. A. 1980. Dung fly struggles – A test of the war of attrition. *Behav. Ecol. Socio.* **7**: 37-44.

Pitnick, S. 1996. Investment in testes and the cost of making long sperm in Drosophila. *Am. Nat.* **148**: 57-80.

Pitnick, S., Henn, K. R. H., Maheux, S. D., Higginson, D. M., Hurtado-Gonzales, J. L., Manier, M. K., Berben, K. S., Guptill, C. & Uy, J. A. C. 2009. Size-dependent alternative male mating tactics in the yellow dung fly, Scathophaga stercoraria. *Proc. R. Soc. B.* **276**: 3229-3237.

Pont, A. C. & Meier, R. 2002. The Sepsidae (Diptera) of Europe. *Fauna Ento. Scand.* **37**: 1-221.

Presgraves, D. C., Baker, R. H. & Wilkinson, G. S. 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. *Proc. R. Soc. B.* **266**: 1041-1047.

Puniamoorthy, N., Kotrba, M. & Meier, R. 2010. Unlocking the "Black box": internal female genitalia in Sepsidae (Diptera) evolve fast and are species-specific. *BMC Evol. Biol.* **10**.

Puniamoorthy, N., Schäfer, M. A. & Blanckenhorn, W. U. 2012. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). *Evolution* (in press).

Puniamoorthy, N., Su Feng Yi, K. & Meier, R. 2008. Bending for love: losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae: Diptera). *BMC Evol. Biol.* **8**: 155.

Puniamoorthy, N., Tan, D., Ismail, M. & Meier, R. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behavior of 27 species of sepsid flies (Diptera: Sepsidae). *J. Evol. Biol.* **22**: 2146-2156.

Reuter, M., Linklater, J. R., Lehmann, L., Fowler, K., Chapman, T. & Hurst, G. D. D. 2008. Adaptation to experimental alterations of the operational sex ratio in populations of Drosophila melanogaster. *Evolution* **62**: 401-412.

Rönn, J. L., Katvala, M. & Arnqvist, G. 2011. Correlated evolution between male and female primary reproductive characters in seed beetles. *Funct. Ecol.* **25**: 634-640.

Rugman-Jones, P. F. & Eady, P. E. 2008. Co-evolution of male and female reproductive traits across the Bruchidae (Coleoptera). *Funct. Ecol.* **22**: 880-886.

Ryan, M. J., Perrill, S. A. & Wilczynski, W. 1992. Auditory tuning and call frequency predict population based mating preferences in the cricket frog, *Acris crepitans*. *Am. Nat.* **139**: 1370-1383.

Sato, T., Hirose, M., Taborsky, M. & Kimura, S. 2004. Size-dependent male alternative reproductive tactics in the shell-brooding cichlid fish Lamprologus callipterus in Lake Tanganyika. *Ethology* **110**: 49-62.

Schäfer, M. A., Misof, B. & Uhl, G. 2008. Effects of body size of both sexes and female mating history on male mating behaviour and paternity success in a spider. *Ani. Behav.***76**: 75-86.

Schneider, J. M. & Elgar, M. A. 2005. The combined effects of pre- and post-insemination sexual selection on extreme variation in male body size. *Evol. Ecol.* **19**: 419-433.

Schneider, J. M., Herberstein, M. E., De Crespigny, F. C., Ramamurthy, S. & Elgar, M. A. 2000. Sperm competition and small size advantage for males of the golden orb-web spider Nephila edulis. *J. Evol. Biol.* **13**: 939-946.

Schulte-Hostedde, A. & Alarie, Y. 2006. Morphological patterns of sexual selection in the diving beetle Graphoderus liberus. *Evol. Ecol. Res.* **8**: 891-901.

Schulte-Hostedde, A. I. & Millar, J. S. 2004. Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (Tamias amoenus): implications for sperm competition and reproductive success. *Behav. Ecol. Socio.* **55**: 272-277.

Schultz, K. S. 1999. *The evolution of mating systems in black scavenger flies (Diptera: Sepsidae).* Doctoral diss., University of Arizona, Tuscon, AZ.

Schuster, S. M. & Wade, M. J. 2003. *Mating systems and strategies*. Princeton University Press, Princeton, NJ.

Schutz, D., Pachler, G., Ripmeester, E., Goffinet, O. & Taborsky, M. 2010. Reproductive investment of giants and dwarfs: specialized tactics in a cichlid fish with alternative male morphs. *Funct. Ecol.* **24**: 131-140.

Servedio, M. R. & Noor, M. A. F. 2003. The role of reinforcement in speciation: Theory and data. *Ann. Rev. Ecol. Evol. Syst***. 34**: 339-364.

Shine, R. & Mason, R. T. 2005. Does large body size in males evolve to facilitate forcible insemination? A study on garter snakes. *Evolution* **59**: 2426-2432.

Short, R. V. 1979. Sexual selection and component parts, somatic and genital selection, as illustrated by man and great apes. *Adv. Stud. Behav.* **9**: 131-158.

Sih, A., Krupa, J. & Travers, S. 1990. An experimental study on the effects of predation risk and feeding regime on the mating behavior of the water strider. *Am. Nat.* **135**: 284-290.

Simmons, L. W. 1988. Male size, mating potential and lifetime reproductive success in the field cricket, *Gryllus bimaculatus* (Degeer). *Ani. Behav.***36**: 372-379.

Simmons, L. W. 2001a. *Sperm competition and its evolutionary consequences in the insects*. Princeton University Press, Princeton, NJ.

Simmons, L. W. 2001b. The evolution of polyandry: an examination of the genetic incompatibility and good-sperm hypotheses. *J. Evol. Biol.* **14**: 585-594.

Simmons, L. W. 2005. The evolution of polyandry: Sperm competition, sperm selection, and offspring viability. *Ann. Rev. Ecol. Evol. Syst.* **36**: 125-146.

Simmons, L. W. & Emlen, D. J. 2006. Evolutionary trade-off between weapons and testes. *PNAS* **103**: 16346-16351.

Simmons, L. W., Emlen, D. J. & Tomkins, J. L. 2007. Sperm competition games between sneaks and guards: A comparative analysis using dimorphic male beetles. *Evolution* **61**: 2684-2692.

Simmons, L. W. & Garcia-Gonzalez, F. 2008. Evolutionary reduction in testes size and competitive fertilization success in response to the experimental removal of sexual selection in dung. *Evolution* **62**: 2580-2591.

Stockley, P., Gage, M. J. G., Parker, G. A. & Moller, A. P. 1997. Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. *Am. Nat.* **149**: 933-954.

Stockley, P. & Purvis, A. 1993. Sperm competition in mammals- A compartive study of male roles and relative investment in sperm production. *Funct. Ecol.* **7**: 560-570.

Teuschl, Y. & Blanckenhorn, W. U. 2007. The reluctant fly: what makes Sepsis cynipsea females willing to copulate? *Ani. Behav.***73**: 85-97.

Teuschl, Y., Reim, C., Meister, B., Egger, J. & Blanckenhorn, W. U. 2010. Strategic Ejaculation in the Black Scavenger Fly Sepsis cynipsea Revisited: Copula Duration as a Function of Sperm Depletion and Body Size. *Ethology* **116**: 1118-1126.

Thüler, K., Bussiere, L. F., Postma, E., Ward, P. I. & Blanckenhorn, W. U. 2011. Genetic and environmental sources of covariance among internal reproductive traits in the yellow dung fly. *J. Evol. Biol.* **24**: 1477-1486.

Tomkins, J. L. & Simmons, L. W. 2002. Measuring relative investment: a case study of testes investment in species with alternative male reproductive tactics. *Ani. Behav.***63**: 1009-1016.

Vahed, K. & Parker, D. J. 2012. The Evolution of Large Testes: Sperm Competition or Male Mating Rate? *Ethology* **118**: 107-117.

Vahed, K., Parker, D. J. & Gilbert, J. D. J. 2011. Larger testes are associated with a higher level of polyandry, but a smaller ejaculate volume, across bushcricket species (Tettigoniidae). *Biol. Lett.* **7**: 261-264.

Watt, P. J., Skinner, A., Hale, M., Nakagawa, S. & Burke, T. 2011. Small Subordinate Male Advantage in the Zebrafish. *Ethology* **117**: 1003-1008.

Wedell, N. 1997. Ejaculate size in bushcrickets: The importance of being large. *J. Evol. Biol.* **10**: 315-325.

Wedell, N., Kvarnemo, C., Lessells, C. K. M. & Tregenza, T. 2006. Sexual conflict and life histories. *Ani. Behav.* **71**: 999-1011.

Wenninger, E. J. & Averill, A. L. 2006. Influence of body and genital morphology on relative male fertilization success in oriental beetle. *Behav. Ecol.* **17**: 656-663.

Zucker, N. & Murray, L. 1996. Determinants of dominance in the tree lizard Urosaurus ornatus: The relative importance of mass, previous experience and coloration. *Ethology* **102**: 812-825.

APPENDIX A:

Overall body size

Head width (FULL)

	df	SS	F	p	
Sex	1	0.025	2.774	0.13	
Continent	1	0.112	2.202	0.179	
Population(Continent)	7	0.436	6.707	**0.008**	
Measurement blocks	18	0.571	3.908	**<0.001**	
Sex * Continent	1	0.186	20.232	**0.002**	
Sex * Population(Continent)	7	0.066	1.154	0.327	§
Error	879	7.131			

Genital morphology (cbrt trans)

(z-transformed body size measures)

Ave. spermathecal volume (FULL)

	df	SS	F	p
Body size	1	0.004	136.82	**<0.001**
Continent	1	<0.001	0.573	0.474
Population(Continent)	7	0.002	9.397	**<0.001**
Measurement blocks	17	0.003	5.591	**<0.001**
Body size * Continent	1	<0.001	2.383	0.167
Body size * Population(Continent)	7	<0.001	2.383	0.07
Error	431	0.012		

Ave. spermathecal volume (n.s removed)

df	SS	F	p
1	0.002	141.071	**<0.001**
1	<0.001	0.876	0.378
7	0.001	9.273	**<0.001**
17	0.003	5.761	**<0.001**
-	-	-	n.s.
-	-	-	n.s.
439	0.012		

(z-transformed body size measures)

Ave. testis volume (FULL)

	df	SS	F	p
Body size	1	0.602	623.27	**<0.001**
Continent	1	0.781	102.83	**<0.001**
Population(Continent)	7	0.053	7.858	**<0.001**
Measurement blocks	18	0.09	5.198	**<0.001**
Body size * Continent	1	0.242	24.765	**0.002**
Body size * Population(Continent)	7	0.069	10.134	**<0.001**
Error	378	0.365		

Pre-copulatory behavioral investment

(z-transformed body size measures)

No of mounts (FULL)

	df	SS	F	p
Body size	1	9.113	7.071	**0.009**
Continent	1	64.32	11.863	**0.011**
Population(Continent)	7	37.96	4.207	**<0.001**
Body size * Continent	1	0.504	0.24	0.639
Body size * Population(Continent)	7	14.71	1.63	0.131
Error	155	199.8		

No of mounts (n.s. removed)

df	SS	F	p
1	3.704	2.813	0.095
1	121.08	12.56	**0.009**
7	67.483	7.322	**<0.001**
-	-	-	n.s.
-	-	-	n.s.
163	214.64		

(z-transformed body size measures)

Precopulatory investment index (FULL)

	df	SS	F	p
Body size	1	9.331	0.546	0.461
Continent	1	1750	7.76	**0.027**
Population(Continent)	7	1579	13.198	**<0.001**
Body size * Continent	1	0.552	0.143	0.717
Body size * Population(Continent)	7	27.12	0.227	0.978
Error	155	2649		

Precopulatory investment index (n.s. removed)

df	SS	F	p
1	1.71	0.104	0.747
1	3267.6	9.983	**0.039**
7	3547.4	30.843	**<0.001**
-	-	-	n.s.
-	-	-	n.s.
163	2678.2		

Post-copulatory behavioral investment

(z-transformed body size measures)

Copulation duration (FULL)

	df	SS	F	p
Male body size	1	202.8	8.414	**0.004**
Female body size	1	190.7	7.911	**0.006**
Continent	1	113.7	1.908	0.21
Population(Continent)	7	417.2	2.472	**0.02**
Male body size * female body size	1	8.156	0.338	0.562
Male body size * Continent	1	2.744	0.082	0.783
Male body size * Population(Continent)	7	234.7	1.39	0.214
Female body size * Continent	1	8.53	2.812	0.137
Female body size * Population(Continent)	7	21.24	0.126	0.996
Male body size * female body size * Continent	1	6.402	0.356	0.57
Male body size * female body size * Population(Continent)	7	125.9	0.746	0.633
Error	137	3303		

Copulation duration (n.s. stepwise removed)

df	SS	F	p
1	1012.9	43.934	**<0.001**
1	232.44	10.082	**0.002**
1	149.13	1.948	0.205
7	535.9	3.321	**0.002**
-	-	-	n.s.
-	-	-	n.s.
-	-	-	n.s.
-	-	-	n.s.
-	-	-	n.s.
-	-	-	n.s.
-	-	-	n.s.
162	3734.8		

(z-transformed body size measures)

Re-mating freq (FULL)

	df	SS	F	p
Body size	1	0.021	0.057	0.812
Sex	1	1.393	0.704	0.556
Continent	1	18.97	18.634	**0.008**
Population(Continent)	5	5.09	2.552	0.163
Body size * Sex	1	7.061	19.07	**<0.001**
Continent * Sex	1	1.964	4.924	0.077
Population(Continent) * Sex	5	1.994	1.077	0.373
Body size * Continent	1	0.077	0.686	0.445
Body size * Population(Continent)	5	0.561	0.303	0.911
Body size * Sex * Continent	1	4.758	6.346	0.053
Body size * Sex * Population(Continent)	5	3.749	2.025	0.075
Error	280	103.7		

Re-mating freq (n.s removed)

df	SS	F	p	
1	0.021	0.057	0.812	
1	7.718	1.421	0.556	
1	22.299	27.016	**0.003**	
5	4.127	2.217	0.053	
1	4.665	12.527	**<0.001**	
-	-	-	n.s.	
-	-	-	n.s.	
-	-	-	n.s.	
-	-	-	n.s.	
2	4.624	6.666	**0.014**	
10	4.625	1.296	0.26	§
280	103.68			

NOTE: Green reflects model presented in paper.
§: N.S. interaction term NOT removed because its needed as error term.

APPENDIX B : http://onlinelibrary.wiley.com/store/10.1111/j.1420-9101.2012.02605.x/asset/supinfo/jeb2605-sup-0002-MovieS1.wmv?v=1&s=ea7e92cbc53f8ac7afa397b10319bc715821f47e

CHAPTER THREE

Comparative analysis of mitochondrial and microsatellite markers suggests incipient speciation among European and North American populations of the widespread fly, *Sepsis punctum*.

Nalini Puniamoorthy, Rudolf Meier, Wolf U. Blanckenhorn & Martin A. Schäfer

ABSTRACT

European (EU) and North American (NA) populations of the dung fly, *Sepsis punctum* (Diptera: Sepsidae) exhibit stark differences in sexual size dimorphism (SSD) as well as differential investment in pre- and post- copulatory traits although morphologically they are the same species. It is unclear if these differences are a result of demographic expansions and/or ongoing processs of selection and diversification. Here, we present a phylogeographic analysis using both a mitochondrial (*COI* gene fragment) and six microsatellite markers to study the underlying genetic structure among twelve *S. punctum* populations (7 EU; 5 NA). We show that allopatric populations of this widespread species exhibit clear genetic differentiation and do not form a panmictic group. The global and continental differences in variation among the independently inherited molecular data is comparably to or even higher than some other widespread Dipteran species (global: Φ_{ST} = 0.390; F_{ST} = 0.218). The *COI* gene fragment yielded a high haplotype diversity (h = 0.625 ± 0.357). Both the maximum parsimony analysis and the haplotype network recovered three geographic clusters: Northern and Central EU, Southern EU and NA. The neutrality tests of Tajima's D and Fu's F_S revealed some negative values but there was no clear evidence of past demographic expansion in these groups. Additionally, admixture analysis of the microsatellite data also recovers distinct geographic clusters (K=8, PP=1.00) that followed a clear spatial differentiation in genetic structure within the continents (Isolation-by-distance: EU - r = 0.789, p = 0.01; NA - r = 0.367, p = 0.025). Thus, the patterns of genetic variation and underlying structure of these allopatric populations of the same species supports the shift in mating system and associated changes in behaviour and SSD that are indicative of incipient speciation in this species.

KEYWORDS

Europe; North America; sepsid flies; incipient speciation; COI; microsatellite

1. INTRODUCTION

Climatic changes during glacial and post-glacial periods, especially around the last glacial maximum, resulted in massive species extinctions, the formation of major refugia and re-colonizations in different parts of the world (Hewitt 1999; Hewitt 2000). Geographic barriers such as mountain ranges and waterways restrained the process of re-colonization of suitable habitats leading widespread lineages evolving in divergent trajectories (Hewitt 1996; Comes and Kadereit 1998; Santucci et al. 1998; Hewitt 2004; Schmitt and Muller 2007). For instance, the phylogeographic history of the European flora and fauna has been largely shaped by the east-west orientation of the Alps, which resulted in diversity hot spots in southern and diversity loss in northern parts of the alps, at least in species adapted to warmer climate conditions (Hewitt 1999; Brito 2007). In contrast to Europe, mountain ranges run north-south in America, which can result in patterns of east-west differentiation that couple with latitudinal variation (Fedorov and Stenseth 2002). Therefore, when studying adaptation and diversification, it is important to consider both the species' evolutionary history as well as ongoing processes of adaptation and selection (Barrowclough et al. 2004; Schmitt and Muller 2007; Pulgarin-R and Burg 2012). Sexual selection, in particular, can play a crucial role in generating pre- or post- zygotic mechanisms of isolation, restricting gene flow among close relatives or even populations (Coyne and Orr 2004). Divergence in direct selection on pre-mating barriers, such as mate preferences and courtship signals, or antagonistic sexual interactions can not only reinforce species boundaries but can evolve even before ecological differentiation, resulting in incipient speciation, either in sympatry or allopatry (Panhuis et al. 2001; Arnqvist and Rowe 2005; Kraaijeveld et al. 2011).

Recent years have witnessed increasing phylogenetic research using molecular information to reconstruct the spatial and temporal patterns of genetic diversification in widespread species. In addition to reconstructing molecular relationships, DNA sequence data can often be used to generate unique haplotypes, joined by mutational steps into networks that can suggest ancestral relationships among closely related taxa and populations (Barrowclough et al. 2004; Brito 2007; Bar Yaacov et al. 2012). When studied in a geographical framework, gene-trees and haplotype networks can have great explanatory power. One particularly fast-evolving marker, the mitochondrial *cytochrome oxidase c subunit I* (*COI*), has been reported to show higher levels of genetic differentiation compared to other genes in the mitochondrial and nuclear genome between species (Wenink et al. 1996; Barrowclough et al. 2004). As a result, COI sequence data are frequently used for DNA taxonomy to re-visit or revise species boundaries among closely related taxa and to discover lineages with significant

geographical information (DeSalle et al. 2005; Hebert and Gregory 2005; Meier et al. 2006).

Most studies on population structure predominantly use microsatellites, multiple neutral loci, in assessing underlying genetic variation. These short tandem repeats are particularly useful in the study of gene flow and geographical structure because of their Mendelian biparental inheritance, codominance, and high levels of polymorphism (Rubinsztein et al. 1999). Nevertheless, these loci often have complicated mutation processes and high mutation rates, and it might be difficult to distinguish alleles that are identical by descent versus state (Estoup et al. 2002). Therefore, it is important to assess population structure using independent genetic markers, and any corroborating or contrasting results from mtDNA and microsatellites, given the different modes of inheritance, can provide unique perspectives into a species' evolutionary history that may be overlooked with merely one source of genetic variance (Brito 2007; Zarza et al. 2011; Pulgarin-R and Burg 2012). Here we present a phylogeographic analysis using both a *COI* gene fragment and microsatellite markers to study the underlying genetic structure among European and North American populations of the widespread dung fly, *Sepsis punctum* (Diptera: Sepsidae).

Sepsis punctum belongs to group of scavenger flies known as Sepsidae, which is a relatively small family with approximately 320 described species across 37 known genera. *S. punctum* has a particularly widespread distribution ranging from North America to Europe and Scandinavia, North Africa and parts of Asia, including Japan and Korea. It is thought to be mesophilic and a generalist that can be found on various types of decaying organic matter, although cow dung is usually the most common breeding substrate (Pont and Meier 2002; Ozerov 2005). Like most insects, sepsid flies generally display female-biased sexual size dimorphism (Blanckenhorn et al. 2007). Interestingly, European populations of *S. punctum* exhibit male-biased SSD whilst in North America the females are larger than the males, although morphologically they are considered the same species. In a recent extensive study, we demonstrated that increased sexual selection on male body size in European *S. punctum* accounts for this geographic reversal of sexual size dimorphism (SSD), while fecundity selection on female body size and overall viability selection on adults is similar between the continents (Puniamoorthy et al. 2012a).

In a follow up study, we also demonstrated that this reversal in SSD is accompanied by differential allocation in pre- vs. post-copulatory traits (Puniamoorthy et al. 2012b). North American females mate rarely, and males invest more in mate acquisition through

frequent mounting attempts and a distinctive pre-copulatory abdominal courtship display. We further observed an east–west gradient in the intensity of male displays in North America, which could potentially play a role in establishing pre-copulatory barriers to gene flow. European populations, on the other hand, display no abdominal courtship and display higher mating rates. Larger males also experience increased lifetime mating success and have evolved relatively larger testes and strong, positive allometry with body size in accordance with sperm competition. Thus, by comparing allopatric populations of the same species we demonstrated a shift in mating system and associated changes in behaviour and SSD that are indicative of incipient speciation in this species (Puniamoorthy et al. 2012b).

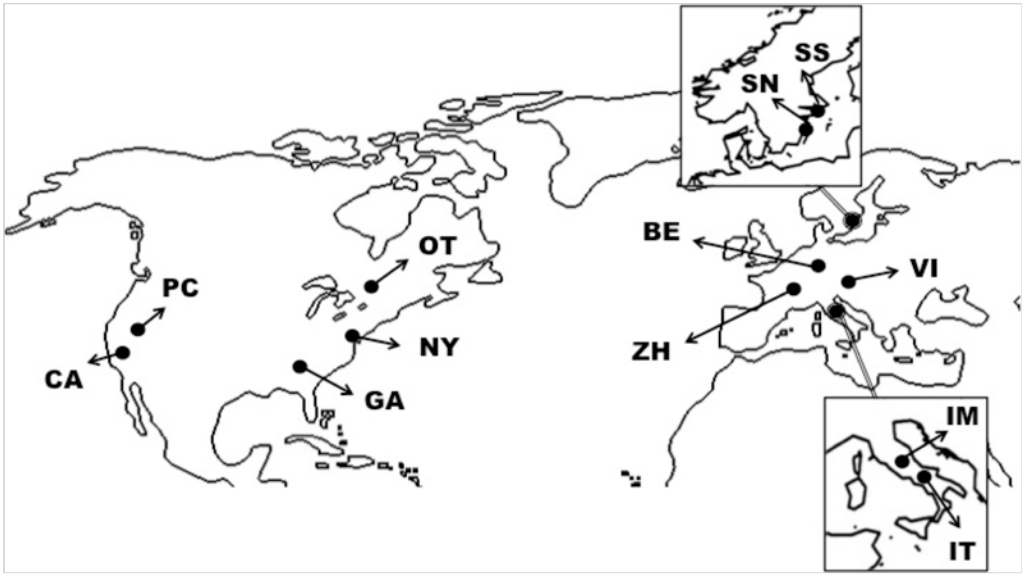
Not much is known about the specific range expansion of *S. punctum*. Although the alpha taxonomy is rather well established, there are still more species being described (Pont and Meier 2002; Ozerov 2005). Most sepsids display elaborate courtship behavior and a recent comparative study of mating behavior across multiple species suggests that mating behavior evolves rapidly in this family and can differ even between closely related taxa (Puniamoorthy et al. 2009). Accordingly, the abdominal courtship observed in North American *S. punctum* populations appears to be autapomorphic to the species so either it was gained in North America or lost in Europe. At this point, it is difficult to assess ancestral relationships among these populations without using molecular tools. Here, using independent *COI* and microsatellite data, we examine the population dynamics in this widespread species by studying the underlying genetic variation among twelve cross-continental populations.

2. METHODS

2.1. Population sampling

Sepsis punctum populations were collected from seven European and five North American sites (Table 1 and Figure 1). Gravid females, caught on and around fresh dung pats in open cow pastures, were transported back to the laboratory in the University of Zurich, and used to establish stock cultures of approximately 10-20 iso-female lines per population. Alternatively, small pots of cow dung were set out in city parks overnight for a few days and shipped back to the laboratory. All fly cultures were housed in separate clear plastic containers, reared in a climate chamber at standardized 24^{o}C, 60% humidity, 14 h light cycle, and were regularly supplied with fresh cow dung, sugar and water *ad libitum.* Single individuals from each line were used for the molecular analysis and 3-5 males from the same line were frozen as voucher specimens. For three populations (CA, GA, NY), cultures were established in the Evolutionary Biology Lab at the National University of Singapore prior to being shipped for study in Zurich.

Figure 1. Populations of *Sepsis punctum* included in this study (black dots). Sampling locations from seven European and five North American sites (geographic co-ordinates given in Table 1).



2.2. DNA extraction; Amplification and sequencing for COI

DNeasy Tissue kits (Qiagen AG, Hombrechtikon, Switzerland) were used to extract DNA from 150 *S. punctum* specimens in total (see Table 1 for details). For the *COI* gene fragment, 4-5 samples per *punctum* population and as well as 31 individuals of four closely related *Sepsis* species from multiple populations were sequenced (*S. cynipsea*, *S. neocynipsea, S. orthocnemis* and *S. fulgens;* see Appendix A). A ca. 740 bp fragment was amplified with diptera-specific *COI* primers (*mtd4*: TACAATTTATCGCCTAAACTTCAGCC and *mtd9*: CCCGGTAAAATTAAAATATAAACTTC) that have been previously used in sepsids (Su et al. 2008). Each reaction used 3µl of the extracted DNA as template, with 0.5µM of each primer, 1 unit *Taq* polymerase (HotStarTaq Master Mix Kit, Qiagen AG, Hombrechtikon, Switzerland) in a total volume of 50µl (manufacturer's buffer). All reactions were run on a DNA Thermal Cycler (Perkin-Elmer Applied Biosytems, Rotkreutz, Switzerland); subjected to an initial 15 min denaturation at 94^{o}C, 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 50^{o}C for 1 min, and elongation at 72^{o}C for 2 min, and a final 7 min elongation step at 72^{o}C. The PCR products were purified using a NucleoSpin Extract II Kit (Macherey-Nagel AG, Oensingen, Switzerland) or a QIAquick PCR Purification Kit (Qiagen AG) following manufacturers' suggested protocols. Following cycle sequencing reactions in total volumes of 20µl (in both directions) and products were cleaning using NucleoSEQ Kit (Macherey-Nagel AG) or via ethanol precipitation and they were sequenced directly on an ABI Prism 3100 Avant Genetic Analyser using Big Dye terminator ver. 3.1 (both Perkin-Elmer Applied Biosystems).

2.3. Microsatellite typing

Four out of the 11 polymorphic microsatellite markers that were isolated and characterized for the closely related *Sepsis cynipsea,* successfully cross-amplified in *S. punctum* (SC_H9, SC_H26, SC_H94, SC_E67; Greminger et al. 2009). Fifteen additional microsatellite primer-pairs were also designed, out of which only two displayed clear allelic amplification (SP_H27 and SP_K11; GenBank Accession FJXXXXXX-X). In total, six neutral markers were tested for polymorphism following the M13-tail PCR method i.e. the addition of a M13 sequence on the 5' end of all 'forward' primers to allow for the incorporation of a fluorescently labelled M13 primer during PCR (Schuelke 2000). Reactions were run with 1µl of extracted DNA, 1 unit PCR buffer (Qiagen AG, containing 1.5mM $MgCl_2$), 0.2mM of additional $MgCl_2$, 0.2mM dNTPs, 0.1µM M13–5'-tailed (TGTAAAACGACGGCCAGT) forward primer, 0.4 µM of reverse primer, 0.3µM 6-FAM-labelled M13(-21) primer, 0.5 unit HotStart Taq polymerase (Qiagen AG) and ddH_2O in a total volume of 20µl. Using the same thermal cycler as earlier mentioned, PCR amplifications were performed with the following conditions: 15 min initial denaturation at 95°C, 34 cycles of 30s denaturation at 94°C, marker-specific annealing temperature for 45s at 72°C, followed by 7 cycles of 30s at 94°C, 30s at 53°C, 45s at 72°C and a final extension step of 30 min at 60°C. The PCR products were separated via a capillary sequencer on a 3730 DNA Analyzer and alleles were scored using GeneMapper 4.0 software (both Perkin-Elmer Applied Biosystems). Microsatellite genotypes were checked twice, independently by two of the authors (NP and MS).

2.4. COI sequence analyses

The *COI* sequences were handled and stored with the help of the Lasergene Program EditSeq (DNAstar Inc., Madison, WI USA). Haplotypes were identified and a minimum-spanning network was constructed based on statistical parsimony at a 95% confidence level using TCS (ver 1.21; (Clement et al. 2000). Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was implemented ARLEQUIN (ver. 3.0; Excoffier et al. 2005), taking into account the number of differences between haplotypes to estimate Φ, analogous to F_{ST} (Weir and Cockerham 1984). The same software was used to calculate Tajima's D and Fu's F_s based on 10000 coalescent simulations, to test for possible past population expansions. For the gene-tree reconstruction, nucleotide sequences from 31 *Sepsis* (outgroup) and 58 *punctum* sequences were aligned using default parameters in Megalign (DNAstar Inc.) together with some previously published *Sepsis COI* sequences (GenBank accession numbers: EU435805, EU435810, EU435815, EU435817). The resulting alignment was free of indels and this data set was subjected to a maximum-parsimony (MP) analysis as implemented in TNT (new tech search, level 55, finding minimum length 10 times; Goloboff et al. 2008). Branch support was assessed via

bootstrapping (250 replicates) with the same options mentioned above. All new *COI* sequences analysed in this study are deposited in GenBank (Accession numbers: EUXXXXXX-XXX).

2.5. Microsatellite analyses

MICROCHECKER, (ver. 2.2.3; Van Oosterhout et al. 2004) was used to check for possible scoring and genotyping errors. The genetic variance in allele frequencies among *punctum* populations was calculated using *F*-statistics according to Weir and Cockerham (1984), with the program MICROSATELLITE ANALYSER (ver. 3.0; Dieringer and Schlotterer 2003). Statistical significance of F_{ST} values was tested by 10 000 permutations of genotypes among populations, not assuming Hardy–Weinberg equilibrium. Sequential Bonferroni correction was also applied to account for multiple testing and an AMOVA was carried out in ARLEQUIN (ver. 3.0; Excoffier et al. 2005) to quantify the amount of genetic variation resulting from differentiation between continents relative to that from population-level variation. To assess population structure, a Bayesian cluster analysis was implemented in STRUCTURE (ver. 2.2; Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009), fitting an admixture model with including a priori sampling information, as is recommended for datasets with limited loci. The putative number of unique clusters or populations (*K)* was set to range from 1 to 12 (100 000 MCMC iterations with a burn in of 25 000). Each value of *K* was run at least thrice to verify repeatability of log-likelihood estimate, the mean of which was used to compute the posterior probabilities of the given *K*, following Bayes' rule and the most likely number of clusters was determined by a probability closest to 1. Finally, to test the relationship between genetic and geographic distances within the continents, isolation by distance (IBD) analyses were performed by regressing linearized Slatkin distances [i.e. $F_{ST}/(1-F_{ST})$] against log-transformed geographical distances between population pairs (Slatkin 1985), the significance of which was determined by a Mantel test in MANTELV2 (Liedloff 1999).

3. RESULTS

3.1. COI gene fragment

Uncorrected pairwise distances across all 12 *Sepsis punctum* populations ranged from 0.002 (SN vs. SS) to 0.034 (SN vs. OT) (Table 3). In Europe, the largest difference was 0.030 (IT vs. SN) and N.America it was 0.013 (PC vs. OT). Global nucleotide diversity was low ($\pi = 0.0025 \pm 0.0016$) and population π ranged from 0.0000–0.0045 (Table 1). Twenty-nine haplotypes were defined in TCS and haplotype diversity ($h = 0.625 \pm 0.357$; see Appendix B) was rather high, ranging from 0 to 1 (Table 1). A clear association between haplotypes and geography was observed with three haplotype-groups that were

recovered separated by more than 8 nucleotide substitutions (North America, Southern Europe, Central and Northern Europe). The two most common haplotypes, *pun_h1* (34%) and *pun_h7* (24%) occurred exclusively among the central and northern European populations while the haplotype, *pun_h2* was only found among the North American populations (Figure 2; Table 1). Figure 2 includes the parsimony network of relationships among all 29, including those that were either private (14%) or singletons (76%) (Table 1). The global Φ_{ST} and the differentiaion between populations was also significant (Φ_{ST} = 0.390, P < 0.0001; Φ_{CT} = 0.070, P < 0.05). This is evidence by the AMOVA showed that although 61% of the variance is attributed to differences within populations, the variance component was not as significant as those components of between population and among continent differences, which explained 32% and 7% percent of the variance respectively (Table 3).

The gene-tree for *COI* included 31 *Sepsis* individuals (outgroups) and recovered a strong monophyletic *punctum* clade with a bootstrap of 99. However, there was no bifurcating population-level differentiation within the continents and no clear signal of ancestral relationship among populations within the species. In fact, the maximum parsimony analysis recovered a trichotomy with the same three clades as per the haplotype network (Figure 2). The neutrality tests of Tajima's D and Fu's F_S revealed some negative values across populations, but there was only one population that was significant (F_S = -1.414; p < 0.05). Overall, there was no clear evidence of past demographic expansion in these groups (Table 1).

Table 1. Population coordinates and analysis of CO1 gene fragment.

		***COI* fragment**					
***Sepsis punctum* populations**	**Coordinates**	N	# Hap.	*h* (SD)	π (SD)	Tajima's D	Fu's F_S
Europe							
SS: Stockholm, Sweden	59.37°N, 18.07°E	5	2	0.400 (0.273)	0.0011 (0.0011)	-0.973	1.040
SN: Nyköping, Sweden	58.67°N, 16.94°E	5	2	0.400 (0.237)	0.0022 (0.0018)	-1.094	2.202
BE: Berlin, Germany	52.45°N, 13.28°E	5	3	0.700 (0.218)	0.0027 (0.0021)	-1.124*	0.644
VI: Vienna, Austria	48.20°N, 16.36°E	5	2	0.600 (0.175)	0.0016 (0.0015)	1.459	1.688
ZH: Zurich, Switzerland	47.40°N, 8.55°E	5	3	0.900 (0.161)	0.0035 (0.0027)	0.000	-0.701
IM: Monte Varchi, Italy	43.53°N, 11.57°E	4	4	1.000 (0.177)	0.0036 (0.0029)	-0.212	-1.414*
IT: Lake Trasimeno, Italy	43.13°N, 12.1°E	4	4	1.000 (0.176)	0.0050 (0.0038)	-0.389	-0.946
North America							
CA: Davis, California, USA	38.54°N, -121.75°E	5	1	0.000	0	0.000	-
PC: Park city, Utah, USA	40.66°N, -111.52°E	5	1	0.000	0	0.000	-
GA: Athens, Georgia, USA	33.96°N, -83.38°E	5	4	0.900 (0.161)	0.0035 (0.0026)	0.562	-0.567
OT: Ottawa, Ontario, Canada	45.42°N, -75.67°E	5	4	0.900 (0.161)	0.0045 (0.0032)	-0.807	-0.128
NY: Manhattan, New York, USA	40.78°N, -73.96°E	5	3	0.700 (0.218)	0.0022 (0.0018)	-1.094	0.276

N: sample size; Hap: haplotypes; h: haplotype diversity; π: Nucleotide diversity (ave. over loci); * p< 0.05

Table 2. Analysis of microsatellite markers. N: sample size; *a*: Ave. number of alleles over all loci; H_O: Observed heterozygosity; H_E: Expected heterozygosity; N_A: Number of alleles per locus.

	Microsatellite markers (ave. over all loci)				SC_E67			SC_H9			SC_H26			SP_H27			SC_H94			SP_K11		
***Sepsis punctum* populations**	N	*a*	H_O (SD)	H_E (SD)	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
Europe																						
SS: Stockholm, Sweden	7	5.33	0.464 (0.278)	0.796 (0.232)	7	0.714	0.964	7	0.500	0.917	2	0.571	0.476	7	0.286	0.976	6	0.714	0.893	3	0.000	0.524
SN: Nyköping, Sweden	10	4.83	0.328 (0.231)	0.705 (0.168)	5	0.600	0.783	7	0.444	0.674	2	0.300	0.550	8	0.125	0.973	4	0.500	0.739	3	0.000	0.511
BE: Berlin, Germany	18	5.67	0.261 (0.215)	0.711 (0.255)	6	0.500	0.798	6	0.467	0.879	4	0.167	0.217	8	0.000	0.912	4	0.375	0.679	6	0.056	0.779
VI: Vienna, Austria	16	4.83	0.369 (0.240)	0.694 (0.204)	5	0.500	0.779	4	0.750	0.780	3	0.250	0.342	7	0.154	0.913	6	0.438	0.775	4	0.125	0.573
ZH: Zurich, Switzerland	18	7.50	0.332 (0.179)	0.725 (0.244)	7	0.611	0.825	10	0.417	0.875	3	0.278	0.263	12	0.188	0.944	6	0.389	0.672	7	0.111	0.770
IM: Monte Varchi, Italy	7	4.50	0.438 (0.303)	0.714 (0.288)	8	0.857	0.964	6	0.571	0.881	3	0.429	0.417	4	0.200	0.925	4	0.571	0.810	2	0.000	0.286
IT: Lake Trasimeno, Italy	12	5.00	0.250 (0.141)	0.671 (0.253)	7	0.182	0.864	5	0.444	0.764	2	0.167	0.303	8	0.125	0.973	4	0.417	0.670	4	0.167	0.451
North America																						
CA: Davis, California, USA	13	2.83	0.048 (0.053)	0.308 (0.157)	3	0.083	0.246	4	0.111	0.576	2	0.000	0.167	2	0.000	0.356	4	0.091	0.350	2	0.000	0.154
PC: Park city, Utah, USA	10	4.83	0.296 (0.175)	0.686 (0.287)	8	0.500	0.917	4	0.500	0.661	2	0.250	0.250	7	0.125	0.955	3	0.300	0.450	5	0.100	0.883
GA: Athens, Georgia, USA	11	4.00	0.269 (0.314)	0.613 (0.323)	7	0.700	0.844	5	0.625	0.777	1	0.000	0.000	4	0.200	0.789	3	0.091	0.505	4	0.000	0.764
OT: Ottawa, Ontario, Canada	14	5.83	0.332 (0.316)	0.655 (0.363)	9	0.818	0.955	6	0.600	0.839	1	0.000	0.000	6	0.273	0.786	3	0.231	0.465	10	0.071	0.887
NY: Manhattan, New York, USA	14	2.00	0.194 (0.225)	0.308 (0.261)	3	0.538	0.593	2	0.400	0.556	1	0.000	0.000	1	0.000	0.000	3	0.154	0.295	2	0.071	0.404
Fixation index			F_{ST}	*p*		F_{ST}	*p*		F_{ST}	*p*		F_{ST}	*p*		F_{ST}	*p*		F_{ST}	*p*		F_{ST}	*p*
Global		14.78	0.218	0.0001	15	0.168	0.0001	23	0.194	0.0001	5	0.104	0.0002	26	0.211	0.0001	8	0.175	0.0001	17	0.362	0.0001
Europe		11.67	0.116	0.0001	13	0.068	0.002	19	0.129	0.0001	5	0.065	0.0217	24	0.071	0.025	8	0.048	0.092	10	0.288	0.0001
North America		9.57	0.280	0.0001	13	0.256	0.0001	13	0.243	0.0001	3	0.059	0.084	15	0.391	0.0001	5	0.313	0.0001	13	0.324	0.0001

Table 3. Upper diagonal – Pairwise F_{ST} based on 6 microsatellite loci; significance indicated by * p <0.05, ** p<0.001, ^{B} sig. after Bonferroni correction. Lower diagonal – Uncorrected pairwise distances for *COI* gene fragment.

			Europe					North America				
	Monte Varchi	Lake Trasimeno	Zurich	Vienna	Berlin	Nyköping	Stockholm	Davis	Park city	Georgia	New York	Ottawa
Monte Varchi	--	0.006	0.163 **^{B}	0.177 **^{B}	0.137 *	0.205 **^{B}	0.171 *	0.462 **^{B}	0.233 **^{B}	0.228 **^{B}	0.458 **^{B}	0.212 **^{B}
Lake Trasimeno	0.010	--	0.149 **^{B}	0.152 **^{B}	0.146 **^{B}	0.201 **^{B}	0.146 *	0.442 **^{B}	0.228 **^{B}	0.229 **^{B}	0.420 **^{B}	0.195 **^{B}
Zurich	0.027	0.028	--	0.083 **^{B}	0.040	0.119 *^{B}	0.096 *	0.337 **^{B}	0.165 **^{B}	0.137 **^{B}	0.324 **^{B}	0.103 **^{B}
Vienna	0.025	0.026	0.004	--	0.098 *^{B}	0.139 *^{B}	0.105 *	0.418 **^{B}	0.175 **^{B}	0.216 **^{B}	0.350 **^{B}	0.157 **^{B}
Berlin	0.026	0.027	0.003	0.003	--	0.100 **	0.098 **	0.340 **^{B}	0.142 *^{B}	0.161 **^{B}	0.325 **^{B}	0.101 *^{B}
Nyköping	0.029	0.030	0.004	0.006	0.004	--	0.016	0.440 **^{B}	0.190 **^{B}	0.224 **^{B}	0.400 **^{B}	0.173 **^{B}
Stockholm	0.028	0.029	0.003	0.006	0.004	0.002	--	0.419 **^{B}	0.164 *^{B}	0.195 *^{B}	0.375 **^{B}	0.130 *^{B}
Davis	0.018	0.021	0.023	0.021	0.022	0.025	0.025	--	0.391 *^{B}	0.356 **^{B}	0.524 **^{B}	0.294 **^{B}
Park city	0.023	0.025	0.026	0.026	0.026	0.026	0.026	0.005	--	0.218 **^{B}	0.389 **^{B}	0.170 **^{B}
Georgia	0.021	0.023	0.026	0.024	0.025	0.028	0.028	0.004	0.008	--	0.346 **^{B}	0.099 *
New York	0.021	0.023	0.026	0.024	0.025	0.028	0.027	0.005	0.009	0.004	--	0.237 **^{B}
Ottawa	0.026	0.029	0.031	0.029	0.031	0.034	0.033	0.012	0.013	0.010	0.010	--

3.2. Microsatellite loci

The six markers showed varying levels of genetic variation among loci and populations. The population from Davis, California had the lowest average allelic richness while Zurich, Switzerland had the highest. Two loci were less variable while the rest were highly polymorphic and the total number of alleles per locus ranged from 5 to 26. All markers showed significant genetic variation among populations with marker-specific global F_{ST} ranging from 0.104 to 0.362 (see Table 2 for details). Overall the differentiation across all *S. punctum* populations as well as within continents was significantly high (F_{ST} : global = 0.218; Europe = 0.116; N. America = 0.280). Pairwise F_{ST} distances within Europe ranged from 0.006 (IM vs. IT) to 0.205 (IM vs. SN) and they ranged from 0.099 (OT vs. NY) to 0.524 (CA vs. NY) in N. America. Surprisingly, this was the largest pairwise difference and not any comparisons between the continents (Table 3). The AMOVA attributed more than 75 % of the genetic variance to differences within populations and 32% to differences within a continent. Interestingly, the continental differences between the microsatellite loci accounted for a similar percentage of the overall variance as did the *COI* (Table 4).

Table 4. AMOVA for mitochondrial and microsatellite data. *COI*: Φ_{ST} = 0.390***; Φ_{SC} = 0.344***; Φ_{CT} = 0.070*; microsat: F_{ST} = 0.218***; F_{SC} = 0.181***; F_{CT} = 0.078***; 10000 permutations; *** p < 0.001.

Source of Variation		d.f.	SS	Variance components	% of variance
Among Continents	*COI*	1	2.081	0.035***	7.02
	microsat	1	35.96	0.184***	7.76
Among Populations	*COI*	10	10.73	0.159***	31.95
	microsat	10	106.49	0.397***	16.7
Within Populations	*COI*	46	14	0.304	61.03
	microsat	139	460.26	1.796***	75.54

Figure 2. Analysis of mtDNA indicates three separate clusters in *Sepsis punctum*. Left – *COI* gene tree for *S.punctum* (in bold) and closely related *Sepsis* species based on maximum parsimony; bootstrap values >70 are given below branch nodes. Right – Haplotype network based on statistical parsimony; area of each circle represents a haplotype proportional to its frequency and colours indicate occurrence in populations and white points are the substitutions.

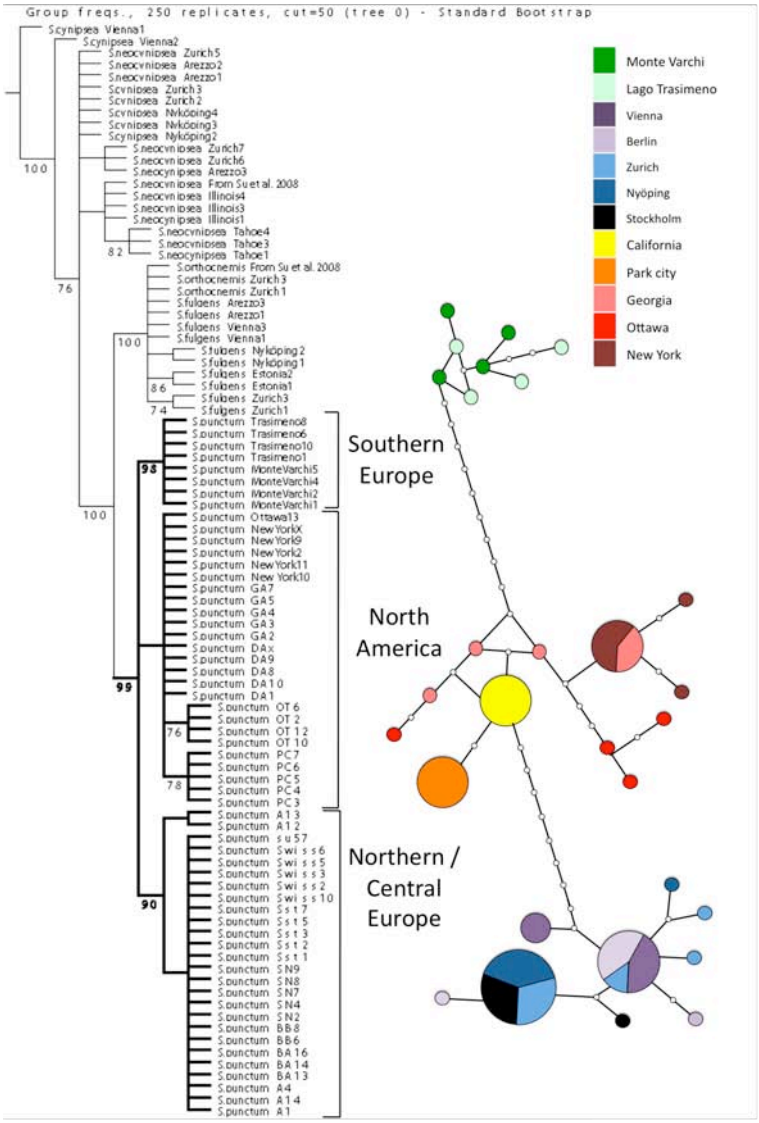
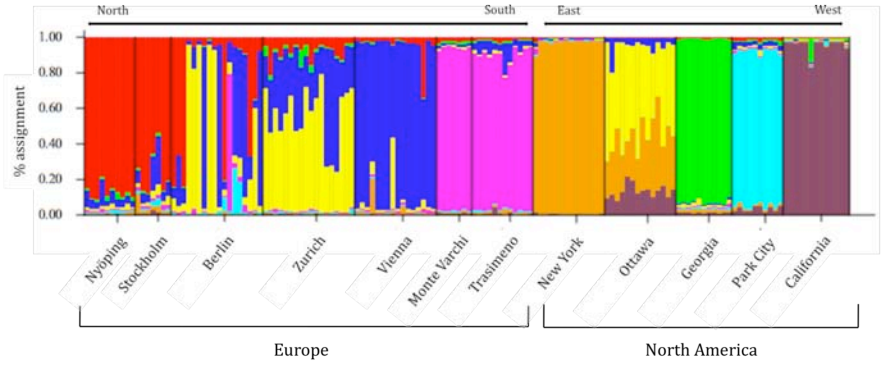
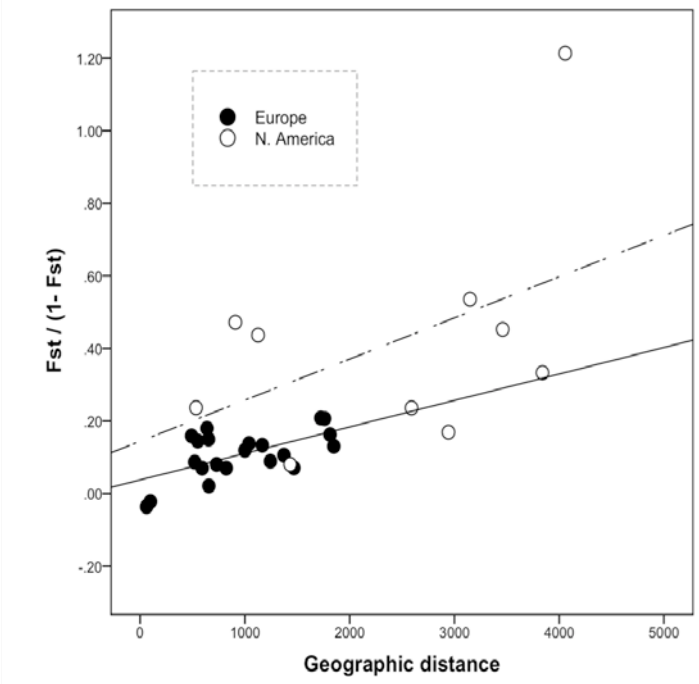


Figure 3. Admixture analysis using microsatellite data of 150 individuals based on 12 *S. punctum* populations. Eight genetically distinct clusters are recovered and indicated by different colours (*K*=8, PP=1.00). Each vertical line represents an individual, partitioned into coloured segments that represent an estimated assignment to a particular cluster.



Bayesian cluster analysis recovered eight genetically distinct clusters based on the twelve *punctum* populations (*K*=8, posterior probability=1.00). Individuals were generally sorted by location, with relatively unique clusters found in Northern Europe (SS, SN), Southern Europe (IM, IT), North Western America (CA, PC) and part of North Eastern America (NY, GA). However, a high degree of admixture was observed Central Europe (ZH, BE, VI) and in one North Eastern American population (OT) (Figure 3). Most notably, in line with genetic separation as suggested by the high pairwise F_{ST}, there was almost no admixture between the extreme west coast and east coast populations in N. America (CA vs. NY). These results are in line with the overall observed genetic variation, given the that approximate geographical distances ranged from 61.45 km (IM vs. IT) to 1847.63 km (SS to IT) in Europe and ranged from 533.91 km (OT vs. NY) to 4057.65 km (CA vs. NY) and there was clear isolation by distance (IBD) in both continents as evidenced by the Mantel test (EU: r = 0.718, p = 0.010; NA: r = 0.367, p = 0.025; Figure 4).

Figure 4. Isolation by distance in Europe (EU) and North America (NA). Regression of pairwise microsatellite differentiation (Slatkin's distance) on geographic distance (km). Mantel test (10,000 permutations) EU: r = 0.789, p = 0.01 (filled circles, solid line); NA: r = 0.367, p = 0.025 (open circles; dashed line).



4. DISCUSSION

This study demonstrates that populations of widespread dung fly, *Sepsis punctum* exhibit distinct genetic variation across North America and Europe based on two sets of independently inherited genetic data. *S.punctum* does not form a panmictic group, instead, showing clear continental differences as well as spatial differentiation in their underlying genetic structure within the continents (Figure 3 and 4). The global and continental differences in variation among both mitochondrial and microsatellite markers in *punctum* (global: Φ_{ST} = 0.390; F_{ST} = 0.218) is comparably to or even higher than some other widespread Dipteran species. For instance, based on mtDNA, haplotype divergence in allopatric populations of European and North American populations of *Drosophila montana* differs by nearly 19 % (Φ_{ST} = 0.187; Routto et al. 2007) while Australian populations of *Aedes vigilax* can differ more than 70 % (Φ_{ST} = 0.7; Puslednik et al. 2012). East African and South Aftrican populations of *Glossina m. morsitans* differ by approximately 13% at neutral loci (F_{ST} = 0.129 to 0.150; Ouma et al. 2007) but there are other commensal species like *Drosophila* (F_{ST} = 0 to 0.07; Caracristi and Schlotterer 2003) or *Scathophaga stercoraria* (F_{ST} = 0.007; Demont et al. 2008) that exhibit limited genetic differentiation in microsatellite markers. The remarkably high population differentiation in *Sepsis punctum* could be a result of limited dispersal, since sepsid flies are not prone to long-range flight (W.U.B. pers. Obs.). Nevertheless, given the behavioral, morphological and life history differences in between the two continents, it is likely that they are undergoing speciation.

4.1. Incipient speciation in North America and Europe

In Europe, we find observe strong isolation by latitudinal distance (Figure 4), which is comparable to the clinal variation observed in various life history traits (Berger et al. submitted). Additionally, there is strong differences in sexual selection acting on male body size and increased investment in post-copulatory traits in Europe (Puniamoorthy et al. 2012a,b). The genetic diversification in Europe is most evident in the clustering of Southern populations (Figure 1 and 3). This north-south differentiation in various plant and animal groups has been attributed to barriers imposed by the east-to-west mountain ranges in Europe such as the Alps, which would have resulted in divergent migration patterns during major climatic events in the past. Patterns of dispersal and expansion in North America, differ significantly. For instance, the Appalachian Mountains define the range expansions in the east whilst the Rocky Mountain the west, both of which run north-south (Hewitt 2000; Fedorov and Stenseth 2002). Directional selection on female mating preferences for male sexual characters can magnify any spatial or geographic variation in male signals (Lande 1982) and sexual selection can reinforce divergence based on mate recognition traits. This is most likely the scenario in North America,

where we observe clear east-west genetic separation that coincides a observed gradient in intensity of pre-copulatory abdominal courtship in *S. punctum* (Puniamoorthy et al. 2012b). When comparing the differentiation within continents, in addition to the geographic barriers, courtship behavior could play a stronger role in reinforcing isolation in North America than variation in body size in Europe. We suggest that restricted gene flow between allopatric populations, has led to the divergence in the male courtship and body size dimorphism, which has clearly been enhanced by sexual selection and that shifts in mating systems are potentially mediating the ongoing process of speciation in *S. punctum* (Puniamoorthy et al. 2012a, b).

4.2. *Range expansions and ancestry in S. punctum*

Sepsids are commonly found on decaying organic matter and it is of particular interest that most of the 'basal' groups are known to use multiple resources ranging from decaying brown algae, horse dung and waterfowl dung. However, a majority of species, including the speciose *Sepsis* genus, specialize on cow dung and clearly the agricultural practices of cattle farming by humans has had a strong influence in shaping species distribution across the globe (Pont and Meier 2002). Although the analysis of mtDNA recovered clearly differentiated clades, we are unable to tease out ancestral relationships between North America and Europe. In fact, the results suggest that evolution of the male-biased size dimorphism could either have been lost in the American populations or gained twice in Europe. Conversely, the pre-mating abdominal courtship was most likely absent in the last common ancestor and was gained once in the American clade. The tests of neutrality (Tajima's D and Fu's F_S) give no indication of sudden past population expansions or recent subdivisions or bottleneck, suggesting that contemporary *S. punctum* estimate expected mutation-drift equilibrium. It has been shown that the expansions from glacial refugia is complex and varies considerably among taxonomic groups (Taberlet et al. 1998). Hence, further studies are required to fully resolve the phylogeography of these flies.

5. REFERENCES

Arnqvist, G., and L. Rowe. 2005. Sexual Conflict. Princeton University Press, Princeton.

Bar Yaacov, D., K. Arbel-Thau, Y. Zilka, O. Ovadia, A. Bouskila, and D. Mishmar. 2012. Mitochondrial DNA Variation, but Not Nuclear DNA, Sharply Divides Morphologically Identical Chameleons along an Ancient Geographic Barrier. Plos One 7.

Barrowclough, G. F., J. G. Groth, L. A. Mertz, and R. J. Gutierrez. 2004. Phylogeographic structure, gene flow and species status in blue grouse (Dendragapus obscurus). Molecular Ecology 13:1911-1922.

Blanckenhorn, W. U., R. Meier, and T. Teder. 2007. Rensch's rule in insects: patterns among and within species. Pp. 60-70 *in* D. J. Fairbairn, W. U. Blanckenhorn, and T. Szekely, eds. Sex, size, and gender roles: evolutionary studies of sexual size dimorphism.

Brito, P. H. 2007. Contrasting patterns of mitochondrial and microsatellite genetic structure among Western European populations of tawny owls (Strix aluco). Molecular Ecology 16:3423-3437.

Caracristi, G., and C. Schlotterer. 2003. Genetic differentiation between American and European Drosophila melanogaster populations could be attributed to admixture of African alleles. Molecular Biology and Evolution 20:792-799.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657-1659.

Comes, H. P., and J. W. Kadereit. 1998. The effect of quaternary climatic changes on plant distribution and evolution. Trends in Plant Science 3:432-438.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Demont, M., W. U. Blanckenhorn, D. J. Hosken, and T. W. J. Garner. 2008. Molecular and quantitative genetic differentiation across Europe in yellow dung flies. Journal of Evolutionary Biology 21:1492-1503.

DeSalle, R., M. G. Egan, and M. Siddall. 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philosophical Transactions of the Royal Society B-Biological Sciences 360:1905-1916.

Dieringer, D., and C. Schlotterer. 2003. MICROSATELLITE ANALYSER (MSA): a platform independent analysis tool for large microsatellite data sets. Molecular Ecology Notes 3:167-169.

Estoup, A., P. Jarne, and J. M. Cornuet. 2002. Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. Molecular Ecology 11:1591-1604.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evolutionary Bioinformatics 1:47-50.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial-DNA restriction data. Genetics 131:479-491.

Falush, D., M. Stephens, and J. K. Pritchard. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. Genetics 164:1567-1587.

Fedorov, V. B., and N. C. Stenseth. 2002. Multiple glacial refugia in the North American Arctic: inference from phylogeography of the collared lemming (Dicrostonyx groenlandicus). Proceedings of the Royal Society B-Biological Sciences 269:2071-2077.

Goloboff, P. A., J. S. Farris, and K. C. Nixon. 2008. TNT, a free program for phylogenetic analysis. Cladistics 24:774-786.

Greminger, M. P., M. A. Schafer, A. Nater, W. U. Blanckenhorn, and M. Krutzen. 2009. Development of polymorphic microsatellite markers for the dung fly (Sepsis cynipsea). Molecular Ecology Resources 9:1554-1556.

Hebert, P. D. N., and T. R. Gregory. 2005. The promise of DNA barcoding for taxonomy. Systematic Biology 54:852-859.

Hewitt, G. 2000. The genetic legacy of the Quaternary ice ages. Nature 405:907-913.
Hewitt, G. M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society 58:247-276.
Hewitt, G. M. 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society 68:87-112.
Hewitt, G. M. 2004. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 359:183-195.
Hubisz, M. J., D. Falush, M. Stephens, and J. K. Pritchard. 2009. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources 9:1322-1332.
Kraaijeveld, K., F. J. L. Kraaijeveld-Smit, and M. E. Maan. 2011. Sexual selection and speciation: the comparative evidence revisited. Biological Reviews 86:367-377.
Lande, R. 1982. Rapid origin of sexual isolation and character divergence in a cline. Evolution 36:213-223.
Liedloff, A.C. 1999. Mantel Nonparametric Test Calculator. Version 2.0. School of Natural Resource Sciences, Queensland University of Technology, Australia.
Meier, R., S. Kwong, G. Vaidya, and P. K. L. Ng. 2006. DNA Barcoding and Taxonomy in Diptera: a Tale of High Intraspecific Variability and Low Identification Success. Systematic Biology 55:715-728.
Ouma, J. O., J. G. Marquez, and E. S. Krafsur. 2007. Patterns of genetic diversity and differentiation in the tsetse fly Glossina morsitans morsitans Westwood populations in East and southern Africa. Genetica 130:139-151.
Ozerov, A. L. 2005. World catalogue of the family Sepsidae (Insecta: Diptera). Zoologicheskie issledovania (Zoological Studies) 8:1-74.
Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. Trends in Ecology & Evolution 16:364-371.
Pont, A. C., and R. Meier. 2002. The Sepsidae (Diptera) of Europe. Fauna Entomologica Scandinavica 37:1-221.
Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.
Pulgarin-R, P. C., and T. M. Burg. 2012. Genetic Signals of Demographic Expansion in Downy Woodpecker (Picoides pubescens) after the Last North American Glacial Maximum. Plos One 7.
Puniamoorthy, N., D. Tan, M. Ismail, and R. Meier. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behavior of 27 species of sepsid flies (Diptera: Sepsidae). Journal of Evolutionary Biology 22:2146-2156.
Puniamoorthy, N., M. A. Schäfer, and W. U. Blanckenhorn. 2012a. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). Evolution 66:2117–2126.
Puniamoorthy, N., W. U. Blanckenhorn, and M. A. Schäfer. 2012b. Differential investment in pre- versus post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae). Journal of Evolutionary Biology 25:2253–2263.
Puslednik, L., R. C. Russell, and J. W. O. Ballard. 2012. Phylogeography of the medically important mosquito Aedes (Ochlerotatus) vigilax (Diptera: Culicidae) in Australasia. Journal of Biogeography 39:1333-1346.
Routto, J., D. Mazzi, K. Van Der Linde, P. Mirol, R. K. Butlin, and A. Hoikakala. 2007. The extent of variation in male song, wing and genital characters among allopatric Drosophila montana populations. Journal of Evolutionary Biology 20:1591-1601.
Rubinsztein, D. C., B. Amos, and G. Cooper. 1999. Microsatellite and trinucleotide-repeat evolution: evidence for mutational bias and different rates of evolution in different lineages. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 354:1095-1099.
Santucci, F., B. C. Emerson, and G. M. Hewitt. 1998. Mitochondrial DNA phylogeography of European hedgehogs. Molecular Ecology 7:1163-1172.

Schmitt, T., and P. Muller. 2007. Limited hybridization along a large contact zone between two genetic lineages of the butterfly Erebia medusa (Satyrinae, Lepidoptera) in Central Europe. Journal of Zoological Systematics and Evolutionary Research 45:39-46.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18:233-234.

Slatkin, M. 1985. Gene flow in natural-populations. Annual Review of Ecology and Systematics 16:393-430.

Su, K. F. Y., S. Kutty, and R. Meier. 2008. Morphology versus Molecules: The phylogenetic relationships of Sepsidae (Diptera: Cyclorrhapha) based on morphology and DNA sequence data from ten genes. Cladistics 24:902–916

Taberlet, P., L. Fumagalli, A. G. Wust-Saucy, and J. F. Cosson. 1998. Comparative phylogeography and postglacial colonization routes in Europe. Molecular Ecology 7:453-464.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535-538.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population-structure. Evolution 38:1358-1370.

Wenink, P. W., A. J. Baker, H. U. Rosner, and M. G. J. Tilanus. 1996. Global mitochondrial DNA phylogeography of holarctic breeding dunlins (Calidris alpina). Evolution 50:318-330.

Zarza, E., V. H. Reynoso, and B. C. Emerson. 2011. Discordant patterns of geographic variation between mitochondrial and microsatellite markers in the Mexican black iguana (Ctenosaura pectinata) in a contact zone. Journal of Biogeography 38:1394-1405.

APPENDIX A :

Species/ Population	Locations	n	Species/ Population	Locations	n
S. cynipsea			*S. fulgens*		
Vienna, Austria	48.20°N, 16.36°E	2	Vienna, Austria	48.20°N, 16.36°E	2
Zurich, Switzerland	47.40°N, 8.55°E	2	Arezzo, Italy	43.47°N, 11.87°E	2
Nyköping, Sweden	58.67°N, 16.94°E	3	Nyköping, Sweden	58.67°N, 16.94°E	2
			Zurich, Switzerland	47.40°N, 8.55°E	2
S. neocynipsea			Tartu, Estonia	58.14°N, 26.91°E	2
Arezzo, Italy	43.47°N, 11.87°E	3			
Zurich, Switzerland	47.40°N, 8.55°E	3	*S. orthocnemis*		
Tahoe, USA	39.09°N,- 120.04°E	3	Zurich, Switzerland	47.40°N, 8.55°E	2
Illinois, USA	41.80°N,- 87.65°E	3			

APPENDIX B :

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CHAPTER FOUR

Cross-continental divergence in male scent? Differences in volatile organic compounds among allopatric populations of the dung fly, *Sepsis punctum*

Nalini Puniamoorthy, Florian P. Schiestl & Wolf U. Blanckenhorn

ABSTRACT

Volatile organic compounds (VOC) play an important role in insect chemical communication and research on various dipteran groups suggest that VOCs can even mediate reproductive isolation among closely related species and allopatric populations. This is an exploratory study of VOCs (including cuticular hydrocarbons) in an emerging model species, *Sepsis punctum* (Diptera: Sepsidae) that is widespread across Europe and North America. These isolated populations not only differ in body size sexual dimorphism but also in pre- and post-copulatory traits. Here, we show that VOC bouquets not only differ between related sepsid species but that the VOCs found in *S. punctum* differ between the continents. We identify 29 compounds, of which 22 have been previously reported as pheromones involved in aggregation, sex and even alarm signals in various insects. Importantly, we report nine putative *punctum*-specific compounds that could be potentially associated with the male osmerteria. These are glandular substance-producing organs that are only found on the male hind tibiae and are involved in copulatory behavior. In particular, three fatty acids and a saturated hydrocarbon, undecane, were only detected in the European populations. Future studies including behavioral assays are needed to detail the significance of these compounds in the sexual selection context.

KEYWORDS

Sepsid flies; male osmerteria; volatile organic compounds; population differentiation; GC-MS

1. INTRODUCTION

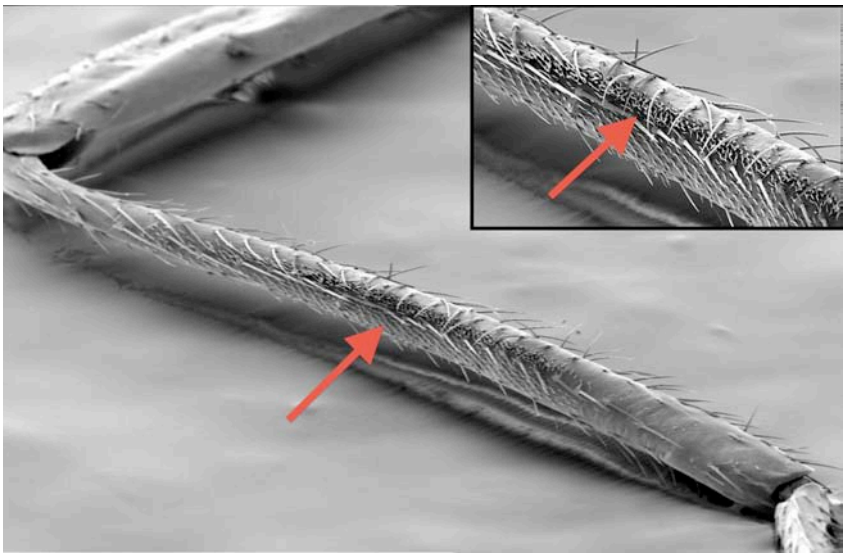
The role of volatile organic compounds (VOC) such as sex pheromones in inter- and intra-specific chemical communication have been well documented across many insect species (Wicker-Thomas 2007). They can play an important role in mediating reproductive isolation because even closely related taxa display divergent pheromonal profiles (Liimatainen and Hoikkala 1998; Hillbur et al. 2000; Hillbur et al. 2001; Hamilton et al. 2002). In Diptera, much of the research on volatile compounds and chemical communication has focused on certain medically and economically relevant groups such as the Psychodidae, Tephritidae, Glossinidae or Drosophilidae (Wicker-Thomas 2007). Research on VOCs and cuticular hydrocarbons among various *Drosophila* lineages in particular has shed light on the evolutionary lability of chemical signals, thus contributing to the current discussion on sexual selection and reproductive isolation (Liimatainen and Hoikkala 1998; Carson 2002; Ferveur 2005; Mas and Jallon 2005). Here we present an initial foray into the VOCs in another important Dipteran model for which sexual selection is suspected of having played a major role in evolutionary diversification, a family of flies known as Sepsidae (Diptera).

Sepsid flies belong to a relatively small family of approximately 320 described species across 37 known genera (Pont and Meier 2002; Ozerov 2005) that display surprising diversity in sexual morphology as well as courtship behavior. Most importantly, sepsids have emerged over the past decades as a model system in sexual selection studies (Eberhard 2003; Blanckenhorn et al. 2004; Muhlhauser and Blanckenhorn 2004; Eberhard 2005; Puniamoorthy et al. 2008; Puniamoorthy et al. 2009; Tan et al. 2010; Teuschl et al. 2010). Of particular interest is the recent work on the widespread *Sepsis punctum* that documents a geographic reversal in sexual size dimorphism (SSD). Males of this species are larger than females in Europe while females are larger than males in North America, and this reversal corresponds to differences in the intensity of sexual selection. In Europe large male body size increases the likelihood of pairing, which is only weakly if at all the case in North America (Puniamoorthy et al. 2012a). This directional reversal in SSD is further reinforced by differential investment in pre- vs. post-copulatory traits In Europe, larger males display a much steeper positive allometry for testes size, implying that they invest disproportionally more in testes (i.e. sperm production) suggesting stronger post-copulatory sexual selection in connection with higher mating rates of females (Puniamoorthy et al. 2012b). This is in sharp contrast to North American populations, which show lower female re-mating rates and a much reduced effect of body size on testes size. Instead, North American males demonstrate an increased investment in mate acquisition prior to copulation, with more mounting

attempts and a distinctive abdominal courtship display that is completely absent in Europe (Puniamoorthy et al. 2012b).

Whereas morphological traits such as body size or secondary sexual traits are clear targets of sexual selection in sepsid flies (Blanckenhorn et al. 2004; Puniamoorthy et al. 2012a; Puniamoorthy et al. 2012b), so far nothing is known about VOCs or their putative role in the reproductive behavior of sepsid flies. Notably, in many sepsid species the apical half of the posterio-dorsal male hind tibiae are often darkened with a setulose patch of what appear to be glandular cells (Figure 1). These are called osmerteria, some sort of substance-producing scent organs that are apparently involved in copulatory behavior. For instance, a comparative study of mating behavior documented that one of the most common copulatory behaviors observed across 23 species involves the male hind tibiae. Males rub their mid legs against their hind tibiae and subsequently rub a certain part of the female. The contact sites of the females differ between species, ranging from the wings to the thorax, abdomen and even the female head (Puniamoorthy et al. 2009; to see a video of the behavior visit http://www.youtube.com/watch?v=ipDkkcwWUs0). Males of European and North American populations of *S. punctum* also have osmeteria on the apical half of their hind tibiae, and the males perform the above-mentioned copulatory behavior by rubbing the female wing and thorax. Here, we first show that VOC bouquets principally differ between closely related sepsid species. We then explore whether there are VOCs associated with the male osmerteria in *S. punctum*, and if they differ among European and North American populations.

Figure 1. SEM of an osmerterium on the hind tibiae of an unidentified male sepsid (Photo credit: Rudolf Meier)



2. METHODS

2.1. Samples and extractions

Laboratory cultures were established for several European (Berlin and Zurich) and North American (Ottawa, Georgia, Utah, California) populations of *Sepsis punctum* as well as two outgroup species, *Nemopoda nitidula* (Berlin, Germany: 52.45°N, 13.28°E) and *Themira biloba* (California, USA: 37.13°N, -121.64°E). The localities of the *S. punctum* populations and the methods for maintaining the cultures are described in Puniamoorthy et al. (2012a; 2012b). Both outgroup species have distinct osmerteria, but only *T. biloba* exhibits the rubbing behavior with the hind leg whilst *N. nitidula* does not (Pont and Meier 2002; Puniamoorthy et al. 2009).

Mating experiments involved virgin flies that were obtained by sexing newly emerged flies within 24 hours of eclosion and subsequently keeping males and females as virgins in separate containers. Mating trials were carried out approximately four days after separation by introducing male and female virgins into group containers. Upon mounting, in copula pairs were removed carefully, placed in glass vials, and frozen immediately at -80 °C for 10 to 15 minutes. Unmated individuals were also frozen under similar conditions and were treated as virgins. The hind legs of both mated and virgin males were dissected. The mid legs of males, which do not possess any osmerteria or glandular cells, were also dissected. All flies were dissected on ice, and individual legs were immediately extracted in glass vials with 50 µl of dichrolomethane and stored at -20 ^{o}C for a maximum of five days prior to analysis using gas chromatography with mass selective (GC-MSD) detection.

2.2. Analysis of sepsid volatile compounds

Extracted samples were analysed with an Agilent 6890N gas chromatograph equipped with a HP-5 capillary column (30 m x 0.32 mm ID; film thickness 0.25 µm), with the inlet temperature kept at 300 ^{o}C (Agilent Technologies, Palo Alto, CA, USA). One µl of extract was injected pulsed spitless at 50 ^{o}C (1 min), and the oven programmed to heat up to 300 ^{o}C at a rate of 10 ^{o}C / min; hydrogen was used as the carrier gas at a flow rate of 2ml/min. All analyses were conducted in two blocks with a control run (1 µl dichrolomethane) each time as well. Compounds were identified by comparison of retention times with those of known reference compounds (Mant et al., 2005). Relative proportions were calculated by dividing the individual amounts by the sum of all the absolute amounts of all compounds using an internal standard (%). The mean relative amounts and standard errors of means of the identified compounds were calculated. Only the most abundant compounds with ≥90% NIST library matches and retention times up to 23 min were considered in this analysis.

2.3. Statistical analysis

Compounds contributing <1 % to the species-specific chemical bouquet were excluded from subsequent analysis. The absolute values of the remaining compounds were log-transformed, and this dataset was further reduced by principal component analysis (PCA) with varimax orthogonal rotation, followed by a MANOVA of the major PCs. Two different analyses were performed: one including all three species, and another with only *S.punctum*. In the former analysis species identity was the explanatory fixed factor, and in the latter continent and mating status (mated or not) were the explanatory fixed factors. All analyses were performed with the software IBM SPSS Statistics version 19.0 (SPSS, Inc., IBM Corporation, Armonk, NY, USA)

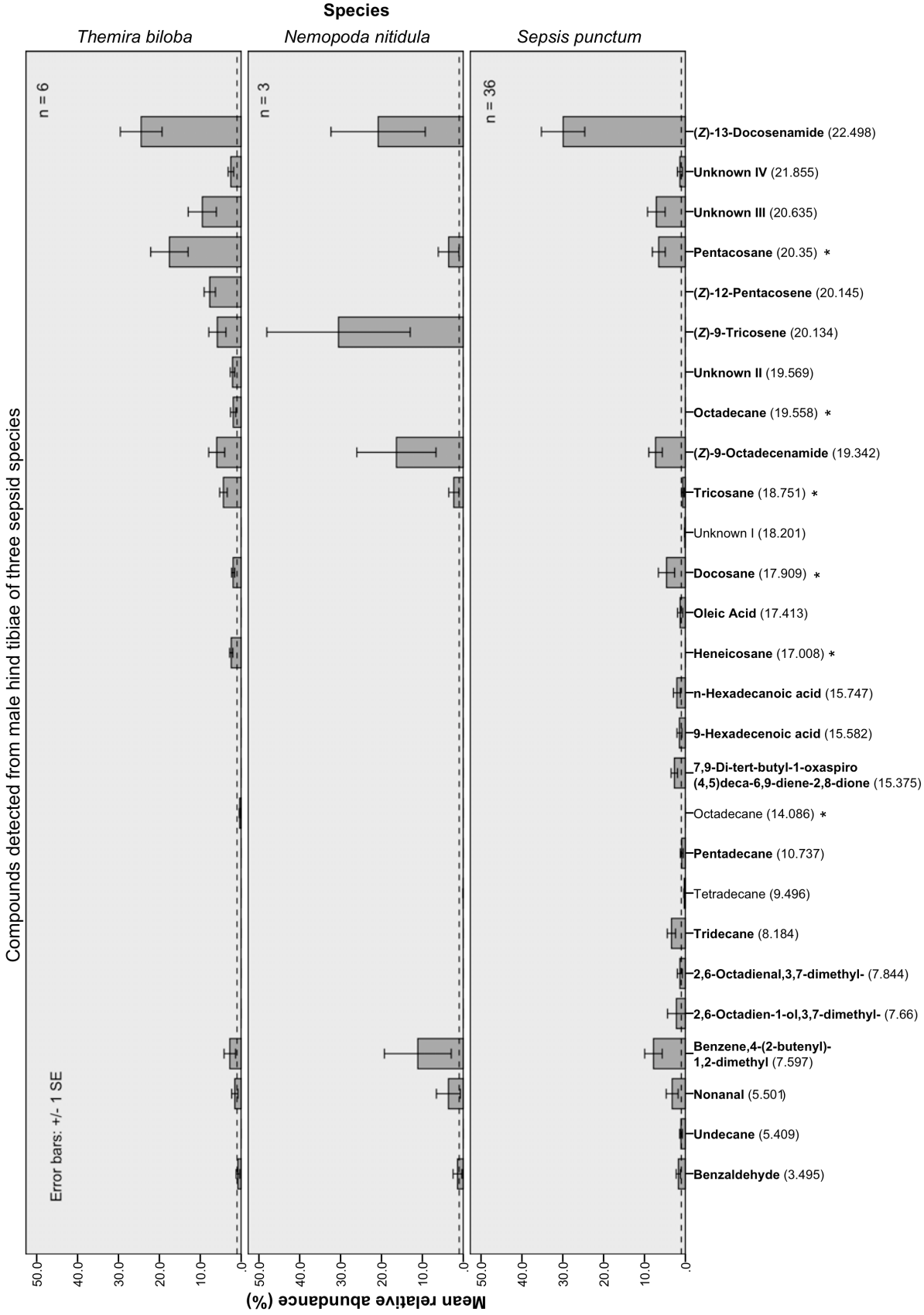
3. RESULTS

3.1. Volatile organic compounds across all three species

A total of 29 compounds were identified based on the leg extractions of 45 males (Table1; Figure 2). Two were excluded from the analysis because they were present in both control runs of the solvent (Phenylacetaldehyde and butylated hydroxytoluene) and six compounds were further confirmed with synthetic reference standards (Octadecane, heneicosane, docosane, tricosane, octadecane, pentacosane). The remaining 27 compounds consisted mainly of straight-chained alkanes (C_{11} to C_{25}), a few alkenes, some aldehydes, alkadienes, fatty acids, plus several unknown polycyclic aromatic hydrocarbons. Out of these compounds, tetradecane, octadecane and one unknown compound (I) were excluded from the overall analysis since their relative species-specific abundance was less than 1 % (threshold indicated in Figure 2).

The PCA of all remaining 24 compounds yielded seven independent principal components (PC) that explained 78.42 % of the total VOC variation across all three species (Table 2). Based on a multivariate analysis of all seven PCs, the three species differed significantly from each other (Wilk's $\boldsymbol{\lambda}$= 0.098; $p < 0.001$). ANOVAs of individual PCs showed they were mainly differentiated by the first, fifth and seventh component (PC1: $F_{2,45}$= 89.27, $p < 0.001$; PC5: $F_{2,45}$= 3.53, $p = 0.035$; PC7: $F_{2,45}$= 5.68, $p = 0.007$), which together account for 37.46 % of the variance in VOCs (Figure 3), and these were strongly loaded mainly with certain alkanes and alkenes (Table 2).

Figure 2. Relative abundance of male hind tibial VOCs across three sepsid species. Retention times given in brackets; compounds NOT in bold were excluded from the overall analysis because they contributed to less than 1 %; * indicates compounds that were confirmed with synthetic reference standards.



3.2. Volatile organic compounds in Sepsis punctum

The analysis of differentiation among European and North American populations was restricted to the 19 compounds that were detected in *S. punctum*. Principal component analysis extracted five PCs, explaining 71.04 % of the total variance in this species. The subsequent MANOVA indicated that overall, the PCs were significantly different both between continents and between mated and virgin males, but there was no clear interaction between the two (Continents: Wilk's **λ** = 0.109, $p < 0.001$; Mating status: Wilk's **λ** = 0.398, $p < 0.001$; Continent by Status: Wilk's **λ** = 0.907, $p = 0.734$). Analysis of individual components showed that 42.74 % of the variation could be attributed to differences between Europe and North America as explained by PCs 1, 4 and 5, which were strongly loaded with one amide, alkanes and fatty acids (Table 2; ANOVAs PC1: $F_{1,34}= 11.27$, $p = 0.002$; PC4: $F_{1,34}= 4.31$, $p = 0.046$; PC5: $F_{1,34}= 15.57$, $p < 0.001$). Mating status was primarily separated by the other two components (PC2: $F_{1,34}= 4.39$, $p = 0.044$; PC3: $F_{1,34}= 18.92$, $p < 0.001$), accounting for remaining 28.3 % variation, which were primarily loaded with alkanes (Figure 4). Of the 19 compounds detected on *S.punctum* male hind legs, nine were completely absent on the mid legs (Table 2, Figure 5). These were mainly alkanes and fatty acids and are possibly associated with the osmerteria, which are only present on the hind legs (Wilk's **λ** = 0.046, $p < 0.001$).

Figure 3. VOC differentiation among three sepsid species for the significant PCs 1, 5 and 7 that explain 23.35, 8.03 and 16.9 % of the variance respectively.

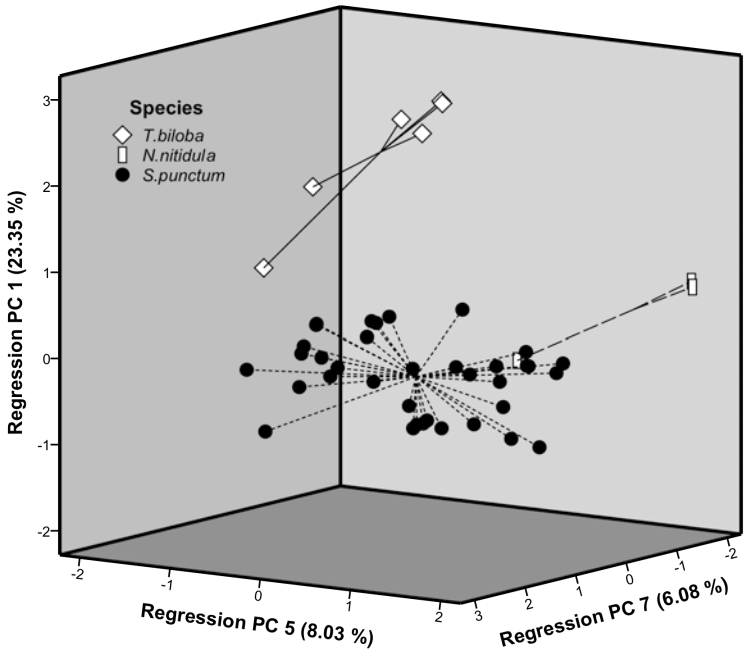


Figure 4. VOC differentiation in *Sepsis punctum* hind leg compounds: [left panel] PCs 1, 4 and 5 explaining 42.74 % of variance among European and North American populations; [right panel] PCs 2 and 3 explaining 28.3 % of variance between mated and virgin males.

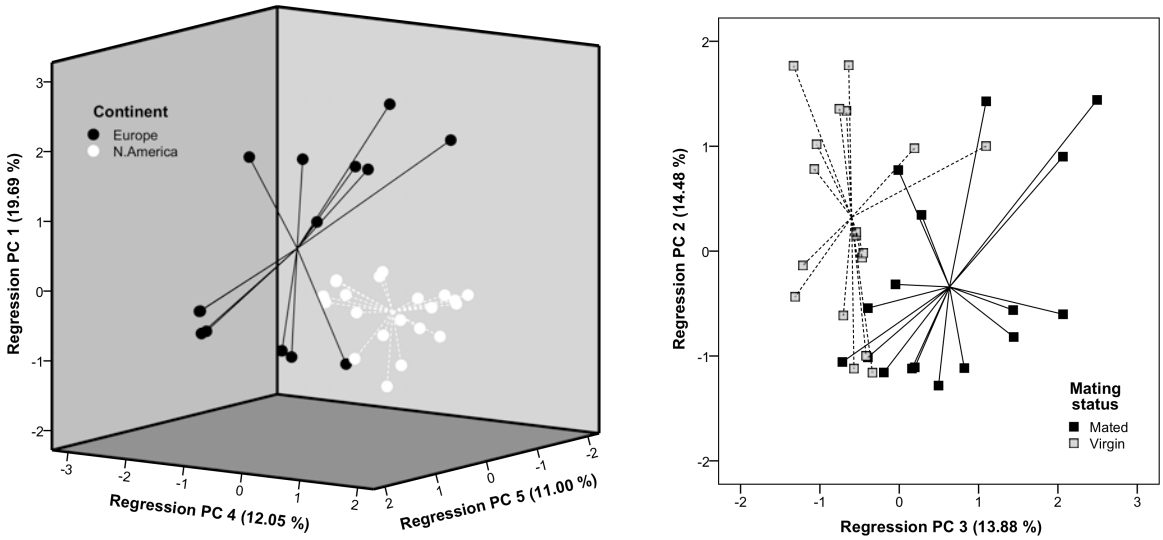


Figure 5. Mid legs and hind leg VOCs detected in *S. punctum*. Blue bars represent 9 compounds that are species-specific.

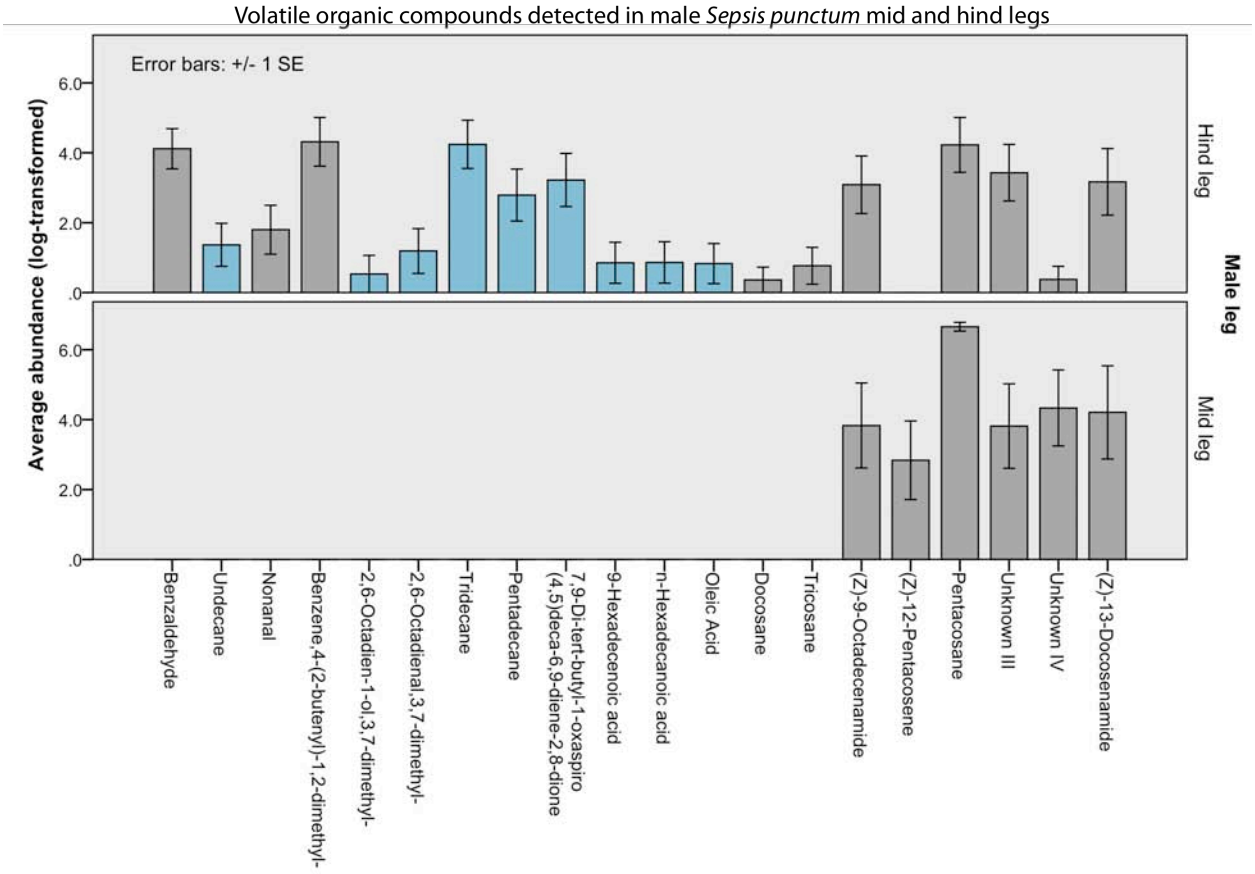


Table 1. Relative abundance (%) of VOCs detected in hind tibial extracts of mated males of three species as well as mid and hind leg extracts of virgin males in *Sepsis punctum*. Sample size is given in brackets beside the species/population name.

Retention Time (min)	Compound	Dichloromethane control (2)	Hind leg						Mid leg			
			Mated males				Virgin males		Virgin males			
			Themira biloba (6)	*Nemopoda nitidula* (3)	*Sepsis punctum*		*Sepsis punctum*		*Sepsis punctum*			
					Europe (8)	N.America (9)	Europe (6)	N.America (12)	Europe (6)	N.America (6)		
3.50	Benzaldehyde	ND	0.79 ± 0.43	1.41 ± 1.08	0.61 ± 0.25	1.93 ± 0.74	0.83 ± 0.32	2.84 ± 1.44	ND	ND		
5.41	Undecane	ND	ND	ND	1.95 ± 0.49	ND	3.32 ± 1.23	ND	ND	ND		
5.50	Nonanal	ND	1.53 ± 0.77	3.64 ± 2.92	1.39 ± 0.37	8.10 ± 5.10	0.33 ± 0.24	2.53 ± 1.97	ND	ND		
7.60	Benzene,4-(2-butenyl)-1,2-dimethyl	ND	2.76 ± 1.41	11.12 ± 8.18	1.64 ± 0.50	8.37 ± 3.43	2.34 ± 1.51	14.68 ± 5.33	ND	ND		
7.66	2,6-Octadien-1-ol,3,7-dimethyl-,	ND	ND	ND	ND	ND	11.26 ± 11.26	ND	ND	ND		
7.84	2,6-Octadienal,3,7-dimethyl-	ND	ND	ND	3.47 ± 2.07	ND	0.13 ± 0.13	1.68 ± 1.17	ND	ND		
8.18	Tridecane	ND	ND	ND	6.73 ± 3.37	ND	3.86 ± 1.59	3.50 ± 1.61	ND	ND		
9.50	Tetradecane	ND	ND	0.06 ± 0.06	0.03 ± 0.03	0.46 ± 0.37	0.03 ± 0.02	0.45 ± 0.26	ND	ND		
10.74	Pentadecane	ND	ND	ND	0.64 ± 0.39	0.03 ± 0.03	0.17 ± 0.10	2.09 ± 1.01	ND	ND		
14.09	Octadecane	ND	0.37 ± 0.09	ND	ND	ND	ND	ND	ND	ND		
15.38	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	ND	ND	ND	0.43 ± 0.13	2.65 ± 1.65	0.91 ± 0.79	5.30 ± 1.75	ND	ND		
15.58	9-Hexadecenoic acid	ND	ND	ND	5.53 ± 2.16	ND	1.13 ± 0.76	ND	ND	ND		
15.75	n-Hexadecanoic acid	ND	ND	ND	8.06 ± 2.96	ND	1.48 ± 1.04	ND	ND	ND		
17.01	Heneicosane	ND	2.41 ± 0.39	ND	ND	ND	ND	ND	ND	ND		
17.41	Oleic Acid	ND	ND	ND	5.05 ± 2.50	ND	0.82 ± 0.61	ND	ND	ND		
17.91	Docosane	ND	1.95 ± 0.38	ND	11.73 ± 6.90	7.83 ± 4.37	0.28 ± 0.18	ND	ND	ND		
18.20	Unknown I	ND	ND	ND	0.16 ± 0.11	0.24 ± 0.13	0.17 ± 0.17	0.15 ± 0.15	ND	ND		
18.75	Tricosane	ND	4.32 ± 0.93	2.30 ± 1.25	2.13 ± 1.21	0.29 ± 0.24	0.55 ± 0.52	ND	ND	ND		
19.34	(*Z*)-9-Octadecenamide	ND	5.97 ± 1.96	16.34 ± 9.69	4.32 ± 1.17	8.32 ± 3.53	4.54 ± 3.75	10.12 ± 3.57	4.61 ± 2.07	10.00 ± 5.35		
19.56	Octadecane	ND	1.94 ± 0.64	ND	ND	ND	ND	ND	ND	ND		
19.57	Unknown II	ND	2.11 ± 0.55	ND	ND	ND	ND	ND	ND	ND		
20.13	(*Z*)-9-Tricosene	ND	5.81 ± 2.09	30.52 ± 17.53	ND	ND	ND	ND	ND	ND		
20.15	(*Z*)-12-Pentacosene	ND	7.67 ± 1.39	ND	ND	ND	ND	ND	3.76 ± 1.55	ND		
20.35	Pentacosane	ND	17.53 ± 4.57	3.58 ± 2.53	5.21 ± 2.46	1.97 ± 1.10	17.16 ± 5.54	4.57 ± 1.82	32.72 ± 9.00	5.73 ± 2.44		
20.64	Unknown III	ND	9.49 ± 3.45	ND	2.73 ± 0.81	0.17 ± 0.17	19.37 ± 6.54	8.09 ± 4.29	18.33 ± 8.16	2.53 ± 2.53		
21.86	Unknown IV	ND	2.49 ± 0.68	ND	4.02 ± 2.21	1.24 ± 0.98	0.00 ± 0.00	0.47 ± 0.47	6.73 ± 2.94	3.61 ± 2.20		
22.50	(*Z*)-13-Docosenamide	ND	24.44 ± 5.10	20.82 ± 11.52	28.31 ± 6.60	42.58 ± 11.25	17.58 ± 11.00	28.67 ± 11.06	28.78 ± ###	46.77 ± 24.21		

Table 2. Loadings of the VOCs on the principal components extracted across all species (7 PCs) and among *S. pucntum* populations (5 PCs). Crosses (†) indicate putative *punctum*-specific osmerteria compounds that are absent on the male mid leg.

Volatile organic compounds	Across three species							Among *Sepsis punctum*					
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 1	PC 2	PC 3	PC 4	PC 5	
Benzaldehyde	-0.19	0.14	0.08	0.08	-0.03	0.53	0.17	0.10	0.15	0.35	0.27	0.21	
Undecane	-0.08	0.48	0.19	-0.36	-0.18	**0.60**	0.08	0.29	0.04	0.33	-0.32	**0.73**	†
Nonanal	0.15	0.08	-0.26	0.26	**0.77**	0.08	0.17	0.13	-0.50	0.59	0.26	-0.31	
Benzene,4-(2-butenyl)-1,2-dimethyl-	-0.01	-0.38	-0.14	0.20	0.31	**0.62**	-0.27	-0.38	-0.24	0.16	0.33	-0.03	
2,6-Octadien-1-ol,3,7-dimethyl-,	0.03	0.42	0.24	0.06	-0.04	-0.25	-0.18	0.44	0.31	-0.11	0.03	-0.08	†
2,6-Octadienal,3,7-dimethyl-	-0.12	0.30	0.48	0.12	-0.24	0.22	-0.07	0.23	0.56	0.03	0.14	0.27	†
Tridecane	-0.32	0.48	0.57	-0.11	-0.31	0.30	-0.12	0.40	0.68	-0.01	-0.10	0.39	†
Pentadecane	-0.36	0.14	**0.78**	0.21	-0.04	-0.13	0.08	0.14	**0.90**	0.12	0.21	-0.20	†
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	-0.56	-0.10	0.10	0.54	-0.05	0.00	0.45	-0.17	0.13	0.15	0.68	-0.18	†
9-Hexadecenoic acid	-0.10	**0.97**	0.07	0.10	0.01	0.06	0.04	**0.96**	0.09	0.14	0.09	0.15	†
n-Hexadecanoic acid	-0.10	**0.97**	0.07	0.11	0.01	0.06	0.05	**0.95**	0.08	0.14	0.10	0.15	†
Heneicosane	**0.90**	-0.12	-0.07	0.04	-0.10	-0.06	0.15	-	-	-	-	-	
Oleic Acid	-0.09	0.97	0.06	0.07	0.02	0.07	0.06	**0.95**	0.08	0.16	0.06	0.14	†
Docosane	0.35	0.07	0.05	0.07	0.20	0.15	**0.85**	0.04	-0.05	**0.84**	0.13	-0.01	
Tricosane	0.59	0.17	0.42	0.03	0.48	0.09	0.22	0.20	0.33	**0.77**	0.01	-0.03	
(*Z*)-9-Octadecenamide	0.24	0.15	0.12	**0.83**	-0.03	0.10	-0.05	0.21	0.16	-0.03	**0.83**	0.18	
Octadecane	**0.85**	-0.06	-0.05	0.12	0.04	-0.08	0.06	-	-	-	-	-	
Unknown II	**0.90**	-0.09	-0.04	0.12	-0.02	-0.05	0.10	-	-	-	-	-	
(*Z*)-9-Tricosene	**0.79**	-0.09	0.05	0.13	0.29	-0.13	-0.21	-	-	-	-	-	
(*Z*)-12-Pentacosene	**0.90**	-0.11	-0.07	0.04	-0.10	-0.06	0.15	-	-	-	-	-	
Pentacosane	0.30	0.01	**0.80**	0.04	-0.12	0.06	0.09	-0.05	0.68	0.27	0.08	0.42	
Unknown III	0.45	0.24	0.24	0.15	-0.73	0.10	0.03	0.19	0.31	-0.29	0.12	**0.80**	
Unknown IV	0.56	-0.14	0.16	0.27	0.02	0.48	0.39	-0.28	0.04	0.58	0.36	0.44	
(*Z*)-13-Docosenamide	0.27	0.25	0.16	0.69	0.36	0.02	0.17	0.31	-0.03	0.37	0.72	-0.05	

4. DISCUSSION

We first show that the volatile organic compounds extracted from the male hind legs differ significantly among the three sepsid species *Themira biloba, Nemopoda nitidula* and *Sepsis punctum*. The main differences are in alkanes and alkenes such as heneicosane, octadecane and 9-(Z)-tricosene, which are known pheromonal compounds found in other insects (Brand et al. 1999; Gotoh et al. 1999; Carpita et al. 2012). European and North American populations of *S. punctum* also differ strongly in some such compounds, and comparisons of the hind against the mid leg compounds suggest that some VOCs are probably specific to the osmerteria, which are substance-producing organs that are involved in copulatory courtship (Puniamoorthy et al., 2009). We show for the first time that sepsid flies indeed produce VOCs, and suggest that these compounds are very likely to play a vital role in chemical communication during reproductive behavior in this family. An obvious next step would be quantitative investigations of sexual selection with regard to particular VOCs in combination with behavioral observations and GC-MS to elucidate the functions of individual compounds produced by the osmerteria.

Of the 29 compounds identified here, 22 have been previously reported to have pheromonal properties in other species. For instance, benzaldehyde and nonanal were established as crucial aggregation pheromones in the common bed bug *Climex lectularius* (Siljander et al. 2008). Nonanal was also identified as an important plant and

floral attractant in shoot flies and mosquitoes (Padmaja et al. 2010; Otienoburu et al. 2012). Pentacosane was involved in predator avoidance in aphids (Nakashima et al. 2004), and (Z)-9-octadecenamide is a contact sex pheromone in shrimps (Zhang et al. 2011). Heneicosane has been detected both as a male-specific compound in skipper butterflies (Omura and Honda 2011) and a female-specific oviposition pheromone in *Aedes aeqypti* (Seenivasagan et al. 2009). Tridecane is part of chemical defense in ants and stink bugs (Brand et al. 1999; Zhao et al. 2012), whilst pentadecane is a known floral attractant in grapevine moths (Tasin et al. 2005). Thus we would also have to investigate potential other functions of the compounds identified here in sepsid flies.

Interestingly, one of the compounds that differed between the three sepsid species studied, (*Z*)-9-tricosene, is the very first sex pheromone recorded and identified in Diptera (Rogoff et al. 1964; Carlson et al. 1971). Together with tricosane, this compound is likely associated with oviposition behavior in female houseflies (*Musca domestica*), since it triggers the aggregation of gravid females and correlates with ovarian maturity (Dillwith et al. 1983). Recently, (*Z*)-9-tricosene has also been identified as a primarily male-produced female attractant in the olive fruit fly *Bactrocera oleae* (Carpita et al., 2012). This compound is a common insect pheromone, and it is even commercially used worldwide in fly bait (Wicker-Thomas 2007). Its function in sepsid flies remains to be investigated in more detail.

Of particular interest here are the nine putative *punctum*-specific osmerteria compounds that were absent on the mid legs (Figure 5; Table 2), out of which four contributed heavily to the differentiation between continents because there were only detected in the European *S. punctum* populations (Figure 2; Table 1). Three of these are fatty acids (9-hexadecenoic acid, n-hexadecanoic acid, oleic acid) and the other is a saturated hydrocarbon, undecane. The latter is actually a common aggressive alarm pheromone in ants that can also be used for interspecific nestmate recognition (Stoeffler et al. 2007; Errard et al. 2008). In male *Tessaratoma* bugs, however, undecane appears to be a potential female attractant that was only found in males (Zhao et al., 2012). It is possible for such a compound to show differential effects in the sexes. For instance, the sex pheromone of the sandfly *Lutzomyia longipalpis* causes female attraction on the one hand and act as an aggregation substance for male conspecifics on the other hand (Spiegel et al. 2005). The three fatty acids found, 9-hexadecenoic acids, n-hexadecanoic acid and oleic acid, have also been detected as both sex and aggregation pheromones in some bees and moths (Takacs et al. 2001; Wang et al. 2005).

To date, more than 125,000 dipteran species have been described, but information on putative chemical communication and pheromones in many of them is still lacking (Yeates and Wiegmann 1999; Wicker-Thomas 2007). In this exploratory study, we have characterized some of the VOCs that may be playing an important role in chemical communication, most likely in the mating context, in sepsid flies. We show that closely related sepsid species differ in their VOC bouquets, and that allopatric populations of *S. punctum* also have diverged in compounds that are potentially associated with the male osmerteria that are involved in copulatory behavior. Future studies including behavioral assays are needed to detail the significance of these compounds in the sexual selection context.

5. REFERENCES

Blanckenhorn, W. U., U. R. S. Kraushaar, Y. Teuschl, and C. Reim. 2004. Sexual selection on morphological and physiological traits and fluctuating asymmetry in the black scavenger fly Sepsis cynipsea. Journal of Evolutionary Biology 17:629-641.

Brand, J. M., L. V. Mabinya, and E. D. Morgan. 1999. Volatile chemicals in glands of the carpenter ant, Camponotus arminius. South African Journal of Zoology 34:140-142.

Carlson, D. A., M. S. Mayer, D. L. Silhacek, J. D. James, M. Beroza, and B. A. Bierl. 1971. Sex attractant pheromone of housefly - Isolation, identification and synthesis. Science 174:76-&.

Carpita, A., A. Canale, A. Raffaelli, A. Saba, G. Benelli, and A. Raspi. 2012. (Z)-9-tricosene identified in rectal gland extracts of Bactrocera oleae males: first evidence of a male-produced female attractant in olive fruit fly. Naturwissenschaften 99:77-81.

Carson, H. L. 2002. Female choice in Drosophila: evidence from Hawaii and implications for evolutionary biology. Genetica 116:383-393.

Dillwith, J. W., T. S. Adams, and G. J. Blomquist. 1983. Correlation of housefly sex-pheromone production with ovarian development. Journal of Insect Physiology 29:377-386.

Eberhard, W. G. 2003. Sexual behavior and morphology of *Themira minor* (Diptera: Sepsidae) males and the evolution of male sternal lobes and genitalic surstyli. Canadian Entomologist 135:569-581.

Eberhard, W. G. 2005. Sexual morphology of male Sepsis cynipsea (Diptera: Sepsidae): lack of support for lock-and-key and sexually antagonistic morphological coevolution hypotheses. Canadian Entomologist 137:551-565.

Errard, C., A. M. Le Guisquet, J. P. Christides, J. L. Mercier, A. Lenoir, and A. Hefetz. 2008. Early learning of volatile chemical cues leads to interspecific recognition between two ant species. Insectes Sociaux 55:115-122.

Ferveur, J. F. 2005. Cuticular hydrocarbons: Their evolution and roles in Drosophila pheromonal communication. Behavior Genetics 35:279-295.

Gotoh, T., K. Nakamuta, M. Tokoro, and T. Nakashima. 1999. Copulatory behavior and sex pheromones in sciarid fly, Lycoriella mali (Fitch) (Sciaridae : Diptera). Japanese Journal of Applied Entomology and Zoology 43:181-184.

Hamilton, J. G. C., R. P. Brazil, D. Campbell-Lendrum, C. R. Davies, D. W. Kelly, F. A. C. Pessoa, and R. G. de Queiroz. 2002. Distribution of putative male sex pheromones among Lutzomyia sandflies (Diptera : Psychodidae). Annals of Tropical Medicine and Parasitology 96:83-92.

Hillbur, Y., M. Bengtsson, J. Lofqvist, A. Biddle, O. Pillon, E. Plass, W. Francke, and E. Hallberg. 2001. A chiral sex pheromone system in the pea midge, Contarinia pisi. Journal of Chemical Ecology 27:1391-1407.

Hillbur, Y., A. El-Sayed, M. Bengtsson, J. Lofqvist, A. Biddle, E. Plass, and W. Francke. 2000. Laboratory and field study of the attraction of male pea midges, Contarinia pisi, to synthetic sex pheromone components. Journal of Chemical Ecology 26:1941-1952.

Liimatainen, J. O., and A. Hoikkala. 1998. Interactions of the males and females of three sympatric Drosophila virilis group species, D-montana, D-littoralis, and D-lummei, (Diptera : Drosophilidae) in intra- and interspecific courtships in the wild and in the laboratory. Journal of Insect Behavior 11:399-417.

Mas, F., and J. M. Jallon. 2005. Sexual isolation and cuticular hydrocarbon differences between Drosophila santomea and Drosophila yakuba. Journal of Chemical Ecology 31:2747-2752.

Muhlhauser, C., and W. U. Blanckenhorn. 2004. The quantitative genetics of sexual selection in the dung fly *Sepsis cynipsea*. Behaviour 141:327-341.

Nakashima, Y., M. A. Birkett, B. J. Pye, J. A. Pickett, and W. Powell. 2004. The role of semiochemicals in the avoidance of the seven-spot ladybird, Coccinella

septempunctata, by the aphid parasitoid, Aphidius ervi. Journal of Chemical Ecology 30:1103-1116.
Omura, H., and K. Honda. 2011. Pungent odor of the adult skipper butterfly Erynnis montanus (Lepidoptera: Hesperiidae). Applied Entomology and Zoology 46:281-286.
Otienoburu, P. E., B. Ebrahimi, P. L. Phelan, and W. A. Foster. 2012. Analysis and Optimization of a Synthetic Milkweed Floral Attractant for Mosquitoes. Journal of Chemical Ecology 38:873-881.
Ozerov, A. L. 2005. World catalogue of the family Sepsidae (Insecta: Diptera). Zoologicheskie issledovania (Zoological Studies) 8:1-74.
Padmaja, P. G., C. M. Woodcock, and T. J. A. Bruce. 2010. Electrophysiological and Behavioral Responses of Sorghum Shoot Fly, Atherigona soccata, to Sorghum Volatiles. Journal of Chemical Ecology 36:1346-1353.
Pont, A. C., and R. Meier. 2002. The Sepsidae (Diptera) of Europe. Fauna Entomologica Scandinavica 37:1-221.
Puniamoorthy, N., K. F. Y. Su, and R. Meier. 2008. Bending for love: losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae : Diptera). Bmc Evolutionary Biology 8.
Puniamoorthy, N., M. R. B. Ismail, D. S. H. Tan, and R. Meier. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behaviour of 27 species of sepsid flies (Diptera: Sepsidae). Journal of Evolutionary Biology 22:2146-2156.
Puniamoorthy, N., M. A. Schäfer, and W. U. Blanckenhorn. 2012a. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). Evolution 66:2117–2126.
Puniamoorthy, N., W. U. Blanckenhorn, and M. A. Schäfer. 2012b. Differential investment in pre- versus post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae). Journal of Evolutionary Biology 25:2253–2263.
Rogoff, W. M., A. D. Beltz, J. O. Johnsen, and F. W. Plapp. 1964. A SEX PHEROMONE IN THE HOUSEFLY, MUSCA-DOMESTICA L. Journal of Insect Physiology 10:239-246.
Seenivasagan, T., K. R. Sharma, K. Sekhar, K. Ganesan, S. Prakash, and R. Vijayaraghavan. 2009. Electroantennogram, flight orientation, and oviposition responses of Aedes aegypti to the oviposition pheromone n-heneicosane. Parasitology Research 104:827-833.
Siljander, E., R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, Cimex lectularius. Journal of Chemical Ecology 34:708-718.
Spiegel, C. N., P. Jeanbourquin, P. M. Guerin, A. M. Hooper, S. Claude, R. Tabacchi, S. Sano, and K. Mori. 2005. (1S,3S,7R)-3-methyl-alpha-himachalene from the male sandfly Lurzomyia longipalpis (Diptera : Psychodidae) induces neurophysiological responses and attracts both males and females. Journal of Insect Physiology 51:1366-1375.
Stoeffler, M., T. S. Maier, T. Tolasch, and J. L. M. Steidle. 2007. Foreign-language skills in rove-beetles? Evidence for chemical mimicry of ant alarm pheromones in myrmecophilous Pella beetles (Coleoptera : Staphylinidae). Journal of Chemical Ecology 33:1382-1392.
Takacs, S., G. Gries, and R. Gries. 2001. Communication ecology of webbing clothes moth: 4. Identification of male- and female-produced pheromones. Chemoecology 11:153-159.
Tan, D., Y. Ang, G. Lim, M. Ibrahim, and R. Meier. 2010. From 'cryptic species' to integrative taxonomy: an iterative pro sequences, morphology, and behaviour leads to the resurrection of *Sepsis pyrrhosoma* (Sepsidae: Diptera). Zoologica Scripta.
Tasin, M., G. Anfora, C. Ioriatti, S. Carlin, A. De Cristofaro, S. Schmidt, M. Bengtsson, G. Versini, and P. Witzgall. 2005. Antennal and behavioral responses of grapevine

moth Lobesia botrana females to volatiles from grapevine. Journal of Chemical Ecology 31:77-87.
Teuschl, Y., C. Reim, B. Meister, J. Egger, and W. U. Blanckenhorn. 2010. Strategic Ejaculation in the Black Scavenger Fly Sepsis cynipsea Revisited: Copula Duration as a Function of Sperm Depletion and Body Size. Ethology 116:1118-1126.
Wang, H. L., C. H. Zhao, and C. Z. Wang. 2005. Comparative study of sex pheromone composition and biosynthesis in Helicoverpa armigera, H-assulta and their hybrid. Insect Biochemistry and Molecular Biology 35:575-583.
Wicker-Thomas, C. 2007. Pheromonal communication involved in courtship behavior in Diptera. Journal of Insect Physiology 53:1089-1100.
Yeates, D. K., and B. M. Wiegmann. 1999. Congruence and controversy: Toward a higher-level phylogeny of diptera. Annual Review Of Entomology 44:397-428.
Zhang, D., J. A. Terschak, M. A. Harley, J. D. Lin, and J. D. Hardege. 2011. Simultaneously Hermaphroditic Shrimp Use Lipophilic Cuticular Hydrocarbons as Contact Sex Pheromones. Plos One 6.
Zhao, D., J. Gao, Y. Wang, J. Jiang, and R. Li. 2012. Morphology and Volatile Compounds of Metathoracic Scent Gland in Tessaratoma papillosa (Drury) (Hemiptera: Tessaratomidae). Neotropical Entomology 41:278-282.

CHAPTER FIVE

Mating behavior evolves faster than morphology: Population divergence in reproductive behavior and sexual dimorphisms in a widespread neotropical fly, *Archisepsis diversiformis* (Diptera: Sepsidae)

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ABSTRACT

Reproductive traits evolve extremely fast and are important in generating sexual isolation and speciation. Comparative work across different taxa suggests rapid diversification in such traits is pivotal in the evolution of species diversity, and that behavioral traits in particular evolve much faster than other types of traits. Here, we present a detailed integrative study of mating behavior and sexual morphology in two populations (Costa Rica & Panama) of the widespread neotropical sepsid fly *Archisepsis diversiformis*. We find that **(i)** despite strong overall similarities in courtship repertoires, some behaviorial elements performed during mating are clearly population-specific, and **(ii)** these populations exhibit clear pre-mating isolation when tested one-on-one. Nevertheless, mass-container population crosses did produce viable F1 offspring after extended exposure to hetero-population individuals. **(iii)** Furthermore, morphometric analysis indicates that the populations differ significantly in wing shape but only moderately in male fore femur shape and not at all in male genital clasper shape. **(iv)** Finally, a comparison of the fast-evolving cytochrome oxidase subunit I (*COI*) gene fragment shows that individuals from Costa Rica & Panama are genetically highly similar, forming a strong monophyletic cluster with uncorrected pair-wise distances only ranging from 0.5-1.6%, thus implying that the behavioral differences between the populations have arisen rather rapidly. We suggest that evolutionary forces are operating strongly on behavioral isolating mechanisms at early stages of diversification in this neotropical fly, and argue that such fine-scaled behavioral work is important when studying incipient sexual isolation and ongoing processes of speciation among widespread species.

KEYWORDS

Sepsid flies; population divergence; speciation; mating behavior, morphometrics, *COI* gene

1. INTRODUCTION

Sexual isolation plays a crucial role in the origin and maintenance of genetic and phenotypic differences among species, and the establishment of such isolation is a pivotal event in the evolution of biological species (Dobzhansky and Mayr 1944; Dieckmann and Doebeli 1999). Ongoing processes of incipient speciation among diverging populations require that groups of individuals acquire means of isolation so as to restrict the gene flow between them and impede hybridization (Seehausen et al. 1997). These can occur during different phases of sexual interactions: pre-mating, post-mating/pre-zygotic, or post-zygotic (Coyne and Orr 2004; Panhuis et al. 2001). Discriminating mechanisms among diverging groups, particularly those resulting in pre-mating barriers, should be favored under selection so as to minimize potential fitness detriments that can arise from secondary contact and gene flow (Groning and Hochkirch 2008). More often than not, behavioral characters are ideal candidates for establishing such reproductive barriers. The importance of non-morphological mating signals in establishing sexual isolation have been documented in various animal groups (Coyne and Orr 2004; Kraaijeveld et al. 2011). Such male-female interactions can range from chemical or olfactory signals, tactile and physical stimulation, complex acoustic songs and calls, to visual cues based on elaborate mating repertoires (Emerson and Ward 1998; Boul et al. 2007; Prohl et al. 2007; Cure et al. 2012). These behavioral traits are often essential for mate recognition and some authors suggest that they are crucial in establishing reproductive isolation, as they evolve faster than morphological structures (Mendelson 2003; Podos et al. 2004; Boul et al. 2007; Podos and Warren 2007; Williams and Mendelson 2010).

Various studies in insects emphasize the role of behavior in generating and maintaining species diversity (Simmons et al. 2001; Vedenina et al. 2007; Puniamoorthy et al. 2009; Luan et al. 2013). For instance, work on certain *Drosophila* species demonstrates rapidly diverging male songs resulting in pre-mating isolation (Gleason and Ritchie 1998; Snook et al. 2005; Klappert et al. 2007), and experimental manipulation of courtship signals in *Gryllus* field crickets shows significant female preferences for conspecific male calls (Gray and Cade 2000; Fitzpatrick and Gray 2001; Gray 2005). Much of the current work is based on acoustic signals while very few studies look at non-acoustic mating behaviors. Exceptions include the early work on blister beetles (Pinto 1977), and more recent studies on water striders (Arnqvist and Rowe 2002), flower weevils (Franz 2003) and antlered flies (Schutze et al. 2007). All these studies indicate that even closely related taxa can have very different and species-specific behavior. Interestingly, some species exhibit inter-population divergence in certain visual and tactile cues, thus facilitating ongoing processes of incipient speciation (Puniamoorthy et al. 2012a, 2012b;

Kim et al. 2012)Chapter 3). Hence, fine-scaled behavioral studies investigating differences in various reproductive traits, especially among diverging populations within a species, are important for understanding the mechanisms involved in sexual selection and reproductive isolation (Klappert et al. 2007; Peretti and Cordoba-Aguilar 2007). Here, we present such an integrative study with a detailed analysis of mating behavior and sexually dimorphic structures in two populations of the widespread neotropical sepsid fly *Archisepsis diversiformis* (Ozerov) (Sepsidae:Diptera).

Sepsids flies occur worldwide, with numerous species having broad distributions spanning more than one continent (Ozerov 2005). They are known to have some of the most extreme sexual dimorphisms in Diptera and are model organisms in studies of sexual selection (Ozerov 2005; Ingram et al. 2008). For instance, the male forelegs are usually adorned with strong cuticular protrusions, modified bristles and/or indentations. These modifications are species-specific and often serve as primary characters for distinguishing species. Sepsids are increasingly studied largely owing to the diversity of courtship behaviors observed across various species. Recent comparative work indicates that mating signals evolve rapidly and that certain visual cues can be species-specific (Puniamoorthy et al. 2008, 2009; Tan et al. 2010, 2011). *Archisepsis diversiformis* is a particularly widespread neotropical species, ranging from Mexico, Jamaica, Costa Rica, Panama, Venezuela, Ecuador, Peru, Brazil to Argentina (Ozerov 2005). Males can be found in high densities waiting for females at dung pats, and as in most sepsids they mount females by clasping their modified fore legs around the female wing bases near female sensory organs (Eberhard 2002; Ingram et al. 2008). *Archisepsis* males often perform courtship displays during copulation, which typically last 20-25 minutes (Eberhard and Huber 1998). Interestingly, Eberhard (2002) noted that in *A. diversiformis* there are differences in male mating behavior between a population from Costa Rica (~1000m, Central Valley near San José) and another from Panamá (~20m, Barro Colorado Island in Lake Gatun) (Figure 1). He indicated that both populations were at least superficially similar in morphology with regard to the male forelegs and claspers, but this was not quantitatively tested (Eberhard and Huber 1998; Eberhard 2001, 2002). We use this ideal opportunity **(i)** to investigate if courtship behaviors in this species are indeed population-specific; **(ii)** to test if they exhibit some degree of pre- or post-mating reproductive isolation; **(iii)** to compare if structures such as the adult wing, male fore legs and genitalia differ between populations using morphometric tools; and **(iv)** to test if these particular populations differ with respect to a particularly fast-evolving mitochondrial barcoding gene, the *cytochrome oxidase c subunit I* (*COI*), that is commonly used for estimating rapid divergence among widespread species (Meier et al. 2006).

2. METHODS

2.1. Sampling and fly cultures

We sampled individuals from San Antonio de Escazu, San Jose, Costa Rica (collected on cattle dung), and on Barro Colorado Island in Panama (collected on monkey droppings), and subsequently established multiple lines for each population (Figure 1). Field-collected males and a subset of male offspring of these females were stored in ethanol for later molecular and morphological studies. Parental cultures were housed in replicate group containers per population, maintained under laboratory conditions (approx. 26 ^{o}C; 60% humidity) over a period of 3 months, and supplied regularly with fresh dung and sugar water.

Figure 1. Populations of *A. diversiformis* used in this study: San Jose, Costa Rica (9.94N, 84.05W) and Barro Colorado Island, Panama (9.15N, 79.85W).

2.2. Body size and development time

We allowed females from parental lines to oviposit for two to three hours and reared the offspring in pots of abundant cow dung. Offspring were raised in a climate chamber, standardized at 24^{o}C, 60% humidity and 14 h light cycle. We recorded the development time from oviposition and measured head width of emergent flies as a standard index of body size using a Leica MS 5 microscope (Leica Microsystems).

2.3. Mating Experiments

Newly eclosed flies were sexed within 24 hours of emergence, and males and females were maintained as virgins in separate containers. Mating trials were carried out approximately four days after separation by introducing one male and one female into a small petri dish. All interactions were video recorded using a SONY hand-held video cam recorder with a 20x magnification lens. Recordings began upon the introduction of both individuals and ended after a successful copulation or after 30-45 minutes if the males did not attempt to mount. The video tapes were digitized using the editing software

iMovies (Apple Computer, Inc., California, USA) and studied frame-by-frame to detail behavioural elements (as per Puniamoorthy et al. 2008; 2009; Tan et al. 2011).

2.3.1. Within-population pairings

Virgin males and females were used to establish mating profiles for each population based on detailed observations of 10-15 successful copulations per population. The interactions were scored qualitatively, i.e. different types of courtship elements were categorized based on behavioral character descriptions previously defined for sepsid flies (Puniamoorthy et al. 2009; Tan et al. 2011). New behaviors were coded as new states, and video clips of individual behaviors are available online on YouTube (Table 1; Character matrix and descriptions in Appendix A).

2.3.2. Between-population pairings

2.3.2.1. One-on-one trials

We conducted reciprocal mating crosses between both populations (female CR x male P; female P x male CR). All focal females were first exposed to a hetero-population male and then a con-population male. Behavioral interactions of all crossing experiments were also video recorded and analysed as described above.

2.3.2.2. Group trials

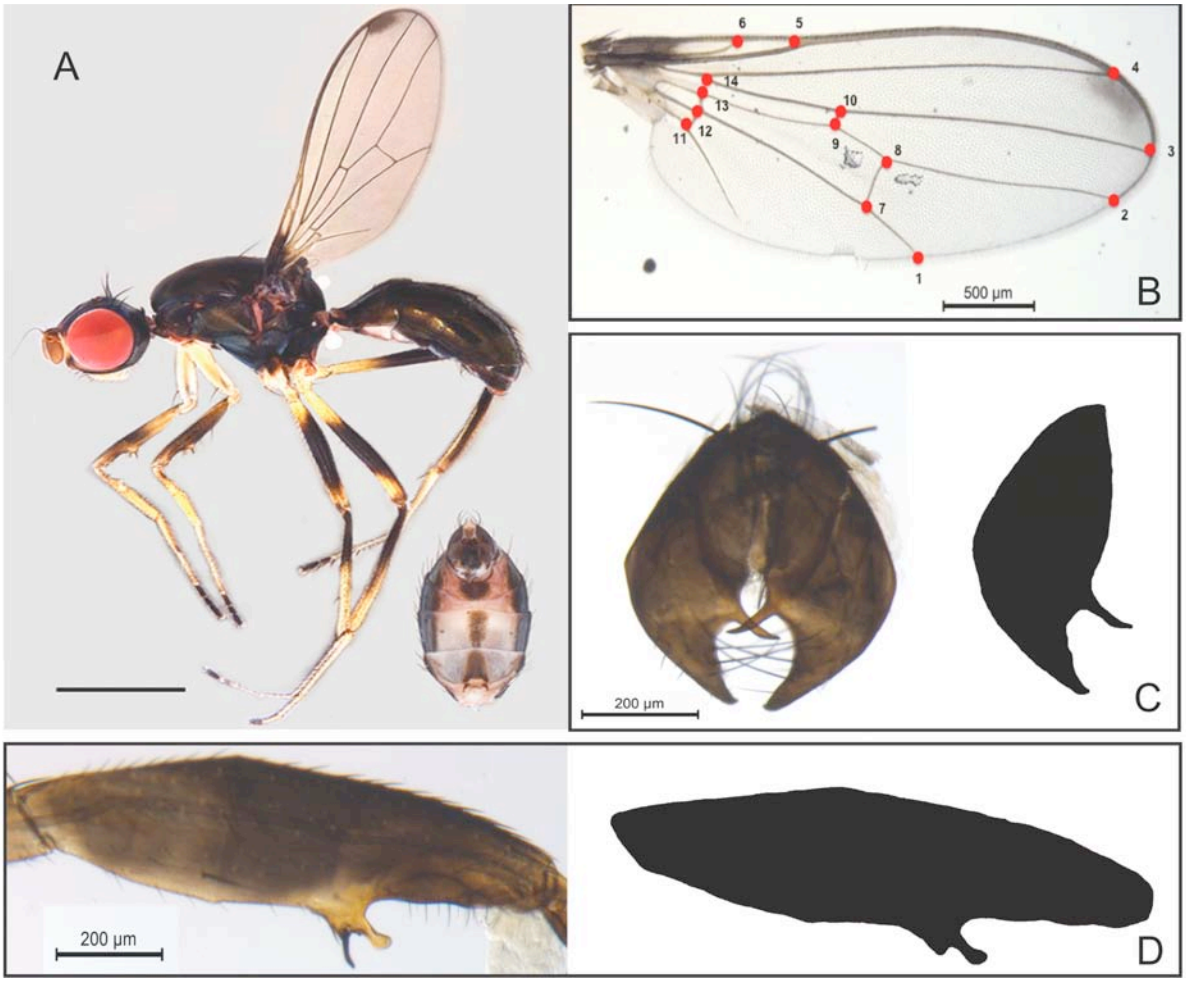
In addition to the one-on-one tests, we also set up four replicate group crossings with five virgin males and five virgin females of the two different populations, assuring prolonged exposure to only hetero-population mating partners. Each group container was provided with fresh dung, sugar and water. All parental adults were killed after two weeks and the dung was left in the containers, maintained in climate chambers under standardized conditions for another four weeks to monitor F1 offspring emergence.

2.4. Morphometric analysis

We dissected and prepared slide mounts of randomly chosen left or right wings, fore leg, mid leg, hind leg and genital claspers (males only) for 30-70 individuals per sex from each population (Figure 2). All structures were imaged at high resolution using a Leica Firecam V. 3.4.1 (Leica Microsystems). To assess wing shape, 14 landmarks were extracted from digital pictures using the computer programs TpsDig v2.10 (Rohlf 2006; see Figure 2B) and procustes transformed using PAST (Hammer et al. 2001), which removes non-shape (i.e. size) variation by centering, scaling and rotating the landmark data to minimize the least-squares deviations among them (Routto et al. 2007). We then performed a principle component analysis (PCA) to test for trait divergence in wing shape among the different populations (Hoffmann and Shirriffs 2002). To assess male

fore femur and clasper shape, the two-dimensional area was determined by capturing the outline as a line drawing based on a digital image of the slide mounted structures (Figure 2C,D). The program SHAPE ver. 1.2. (Iwata and Ukai 2002) was then used to generate elliptical Fourier descriptors (EFD; Kuhl and Giardina 1982), which were again subjected to PCA in SHAPE.

Figure 2. *Archisepsis diversiformis*, Costa Rica. (A) Habitus picture [Credit: Sepsidnet. 2012. World wide web electronic publication. sepsidnet-rmbr.nus.edu.sg ver. 10/2012)]; (B) Wing: Fourteen landmarks, each one defined by *x, y* variables; (C) Male clasper: Left- digital image; Right- line drawing based on part of image; (D) Male fore femur: Left- digital image; Right- line drawing based on image.



2.5. Statistical analysis

All analyses were performed with the software IBM SPSS Statistics version 19.0 (SPSS, Inc., IBM Corporation, Armonk, NY, USA) except for the PCAs of wing (in PAST) and foreleg and genital shape (in SHAPE). To test for significance for overall body size and development time variables as well as for the wing-specific principal components (PCs), we used ANOVA and MANOVA (respectively) with population and sex as explanatory factors. For the PC shape analysis of male forelegs, claspers and behavior traits, we used MANOVA to test for population differences in these variables.

2.6. COI gene fragment amplification and sequence analysis

We used DNeasy Tissue kits (Qiagen AG, Hombrechtikon, Switzerland) to extract DNA from 6 wild-caught specimens from each population. The particulars of the primers used, as well as the PCR reaction conditions, have been previously detailed (Puniamoorthy et al. in prep.; Chapter 3). The *COI* sequences were handled and stored with the help of the Lasergene Program EditSeq (DNAstar Inc., Madison, WI USA), and pair-wise distances were generated with MEGA5 (Molecular Evolutionary Genetics Analysis; Tamura et al. 2011). For the phenetic reconstruction, nucleotide sequences from 12 *A. diversiformis* individuals were aligned using default parameters in Megalign (DNAstar Inc.) together with six previously published *Archisepsis* and *Microsepsis COI* sequences (GenBank accession numbers: EU435774-77, EU435794 & EU435795). The alignment was free of indels, and this data set was subjected to a neighbor-joining tree reconstruction method (nucleotide substitution model: Kimura 2- parameters). Branch support was assessed via bootstrapping (1000 pseudo-replicates) with the same substitution model. All new *COI* sequences analysed in this study are deposited in GenBank (Accession numbers: EUXXXXXX-XX).

3. RESULTS

3.1. Overall body size and development time

The overall body size based on head width differed significantly between the sexes with both populations showing a strong female-biased sexual size dimorphism (Figure 3; sex effect: $F_{1,151} = 53.14$, $P < 0.001$) . However, there was very little difference between the populations and no significant interaction with respect to body size (population effect: $F_{1,151} = 1.94$, $P = 0.166$; sex by population: $F_{1,151} = 0.234$, $P = 0.630$). Development time also did not vary between populations or the sexes, although the interaction was nearly significant showing development time differences between the sexes for the Costa Rica but not the Panama population (Figure 3; population effect: $F_{1,150} = 0.003$, $P = 0.957$; sex effect: $F_{1,150} = 0.957$, $P = 0.330$; sex by population: $F_{1,150} = 2.97$, $P = 0.088$). This implies that the body size dimorphism is largely due to accelerated growth rather than longer development of females, a common pattern in animals (Blanckenhorn et al. 2007).

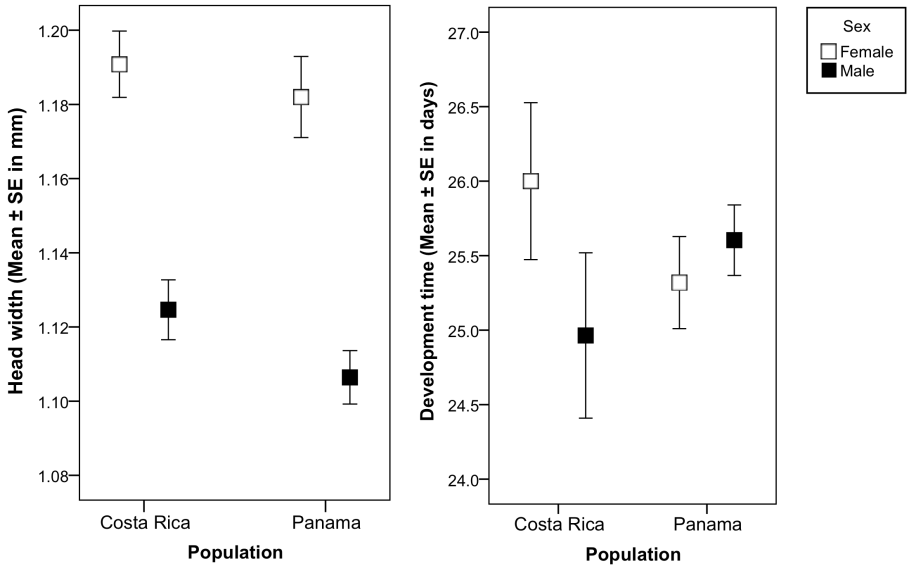
3.2. Mating behavior of A.diversiformis

3.2.1. Mating experiments within populations

On the whole, both populations had similar mating frequencies (binary logistic model on 1/0 data- population effect: $\chi^2 = 0.028$, $p = 0.866$) and did not differ in average copulation duration (population effect: $t_{24} = 0.011$, $p = 0.992$; Table 1). The overall mating behavior of *A. diversiformis* can be characterized by the presence of certain

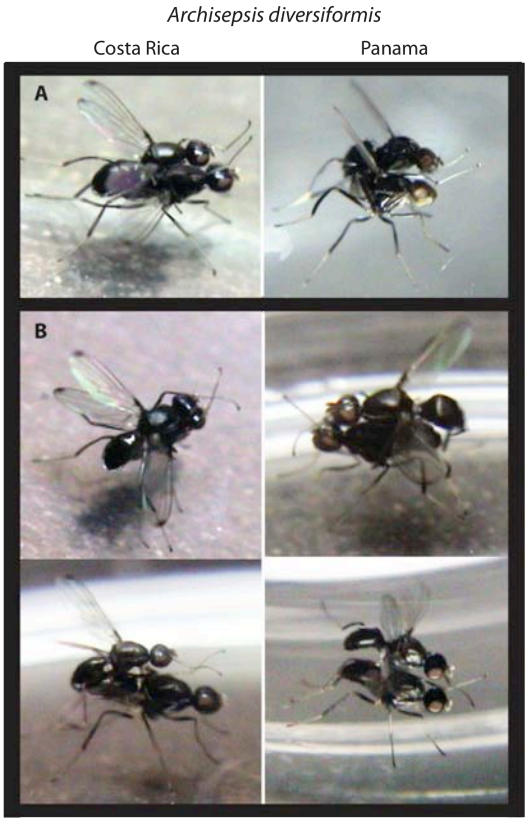
behaviors that were present in both populations (Table 1; see Appendix A,B for detailed character descriptions and table of individual behaviors). For instance, in both populations males repeatedly lowered their surstyli to stimulate the female posterior abdomen prior to copulation (Table 1, clip 1). Males also used their mid legs and hind legs to 'rub' or 'tap' different parts of the female during both the pre-copulatory and copulatory phases. Video evidence for these behaviors is available online on YouTube (Table 1; http://www.youtube.com/user/sepsidbehavior2013). Of particular interest is a distinctive male mid-leg behavior: *A. diversiformis* males extend both their mid tarsi forward and at an angle away from the female ocelli (eyes), a display that appears to be specific to this species (Figure 4A; see Table 1, clip 1). This behavior can be observed in both populations intermittently throughout courtship and copulation when mounted.

Figure 3. Mean body size and development time in both populations of *A. diversiformis*; Left- distinct female-biased SSD (n=152); Right- moderate albeit not significant difference in egg to adult development time between Costa Rica and Panama populations (n=151).



However, the populations do differ with respect to a few characters. For instance, in addition to the above-mentioned mid-tarsal display, males intermittently curl their tarsi towards the female head. A major difference is that in Panama males only curl one tarsus whilst males from Costa Rica curl both (Figure 4B; Table 1, clip 2). Another striking difference is that males from Panama move their mid legs repeatedly away from the female head. This is a pre-copulatory mid leg movement resembling a 'swimming' action that is absent in the Costa Rican population (clip 3). Most copulatory behaviors are observed in both populations (clip 4), with the exception of a female behavior in Costa Rica. Here, females intermittently 'rub' their hind legs against the male hind legs (especially the hind tibiae), which is rare among females from Panama (clip 5).

Figure 4. (A) Mid leg display unique to *A. diversiformis* observed in both populations. (B) Mid tarsal curl performed differently: (left) both tarsi curled in Costa Rican males, (right) single tarsus curled in Panama males.



3.2.2. Crossing experiments between populations

3.2.2.1. One-on-one trials

All focal females did not mate when first exposed to a hetero-population male but a significant number copulated when they were subsequently introduced to a con-population male (Table 2). A binary logistic model on the mating outcome (1/0 data) shows that this pattern holds true for both populations (Identity of test male: χ^2 = 18.40, $p < 0.001$; Population effect: $\chi^2 = 0.29$, $p = 0.590$). Interestingly, in most of the trials, regardless of population, males also made fewer attempts to mount hetero-population females (Mounting attempt (1/0 data)- Identity of focal female: $\chi^2 = 14.93$, $p < 0.001$; Population effect: $\chi^2 = 0.038$, $p = 0.846$). Nevertheless, when they did mount, hetero-population males (i) were shaken or 'kicked' off by females and/or (ii) they dismounted immediately or soon after performing some pre-copulatory courtship (Appendix B; clip 6: http://youtu.be/whSv4O1DFl8).

3.2.2.2. Group trials

In the group set up with prolonged exposure to hetero-population mating partners, eggs, larvae and F1 offspring adults were present in of all eight containers, indicating that

females from both populations did eventually mate with hetero-population males, with viable F1 being produced in all replicates.

Table 1. Summary of reproductive behaviors observed in both populations of *A. diversiformis.*

	Costa Rica	Panama	Supporting video links	
Observed behavioral characters				
Precopulatory				
Male mid leg display at an angle from female ocelli Surstylus stimulation Male hind leg tap	Observed in both populations		http://youtu.be/eMX2aQRWQ9o	Clip 1
Male mid leg tarsi curl towards female head	Both tarsi	Single tarsus	http://youtu.be/PZghFz6zOic	Clip 2
Male mid leg 'swim' away from female head	Absent	Present	http://youtu.be/YOznwZelnv4	Clip 3
Copulatory				
Male mid leg display at an angle from female ocelli Male mid leg extension 'balancing' Substance transfer (from male hind leg to female wing) Male hind leg tap Male fore leg wing release and interaction with female	Observed in both populations		http://youtu.be/-ApT6yH_BXQ	Clip 4
Female uses hind legs to rub male hind legs	Present	Rare	http://youtu.be/taO9D7FqoP4ed	Clip 5
Mating frequency (Successful copulations/Total trials)	14/30	12/27		
Copulation duration (Ave. ± SD in min)	28.65 ± 5.00	28.63 ± 4.95		

Table 2. One-on-one crossing experiments between Costa Rica and Panama populations. First male tested is a hetero-population followed by a con-population male.

Focal female	Test male	No. of trials	No. of copulations
Costa Rica (CR)	1st : PAN	16	0
	2nd : CR	16	6
Panama (PAN)	1st : CR	16	0
	2nd : PAN	16	8

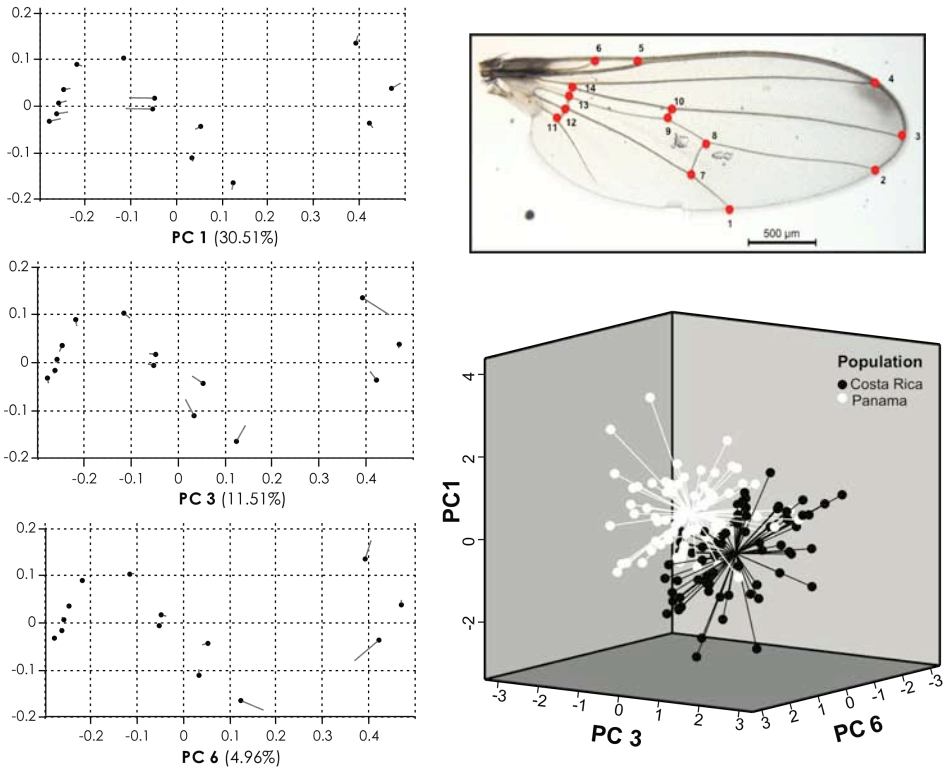
3.3. Morphometric analysis

3.3.1. Wing shape

The landmark analysis of wing shape was based on 148 wing images (sample sizes, male: CR=44; PAN=48; female: CR=26; PAN=30). A PCA of procustes-transformed landmark coordinates extracted six significant PCs that cumulatively explained 80.25% of the total variation. A subsequent MANOVA detected significant differentiation between the populations, influenced strongly by changes in the internal landmarks of the wings (Figure 5; PC1: landmarks (LM) 9 & 10) as well as changes on the edges of the wings (PC3: LM 1 & 4; PC6: LM 1, 2 & 4). There were also sex differences in wing shape but there was no interaction between the two (Populations: Wilk's $\boldsymbol{\lambda}$ = 0.546, $p < 0.001$;

Sex: Wilk's **λ** = 0.405, p < 0.001; Population by sex: Wilk's **λ** = 0.934, p = 0.209; Appendix C).

Figure 5. Analysis of wing shape. Left- Landmark displacements (+ 0.1 vector deviation of PC) are shown for PC 1, 3 & 6 that account for significant variation in wing shape between populations. Right- Wing with 14 landmarks (top); Plot of the population variation in the three axes (PC1, 3 & 6) (bottom).



3.3.2. Fore femur shape

A total of 45 harmonics were used to extract 177 elliptical Fourier descriptors (EFD; *x, y* dimensions and sine cosine components for each harmonic) from 146 fore femur images (sample size: CR=73; PAN=73). These were analysed with PCA, resulting in 15 main axes accounting for 94.34% of the total variation. The first three explained 64.52% of the differences in fore femur shape, illustrating the strong individual variation in the thickness of the femur and femoral protrusion (Figure 6; Appendix C). However, these were not significantly different between the populations. The most significant between-population variance was explained by PC4 (9.74%), PC5 (5.12%), PC6 (2.90%) and PC10 (1.33%), but these cumulatively contributed less than 20 percent of the total variation (Appendix C).

3.3.3. Genital (clasper) shape

As for the fore femur analysis, 177 EFDs were extracted from 45 clasper images (sample size: CR=23; PAN=22). The PCA extracted 11 axes explaining 96.63% of the total variation. Much of the variation in thickness or narrowness of the outer and inner

processes of the clasper was captured by the first 3 PCs (Figure 7). Nevertheless, a MANOVA of all PCs suggests that despite this strong individual variation in clasper shape, there are no significant differences between Costa Rica and Panama males (population effect: Wilk's λ = 0.668, p = 0.182; Figure 7; Appendix C).

Figure 6. Male fore femur shape. Left- Shape outlines corresponding to the mean (in black) and the standard deviations in both directions (in grey) are shown for the first three PCs that individually account for at least >10% of the total variation in shape. Right- Plot of these axes showing little to no differentiation between populations.

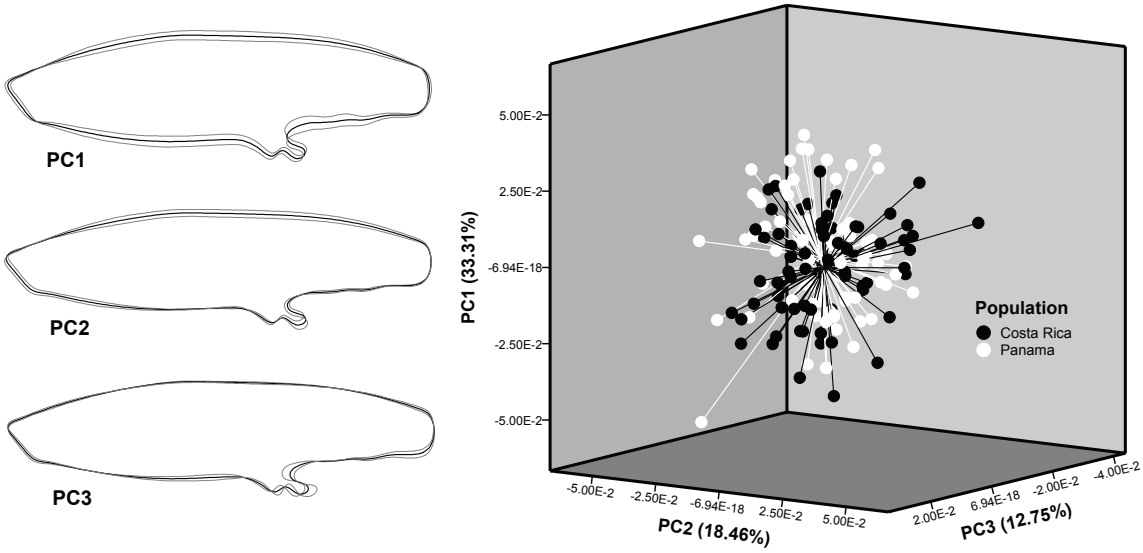
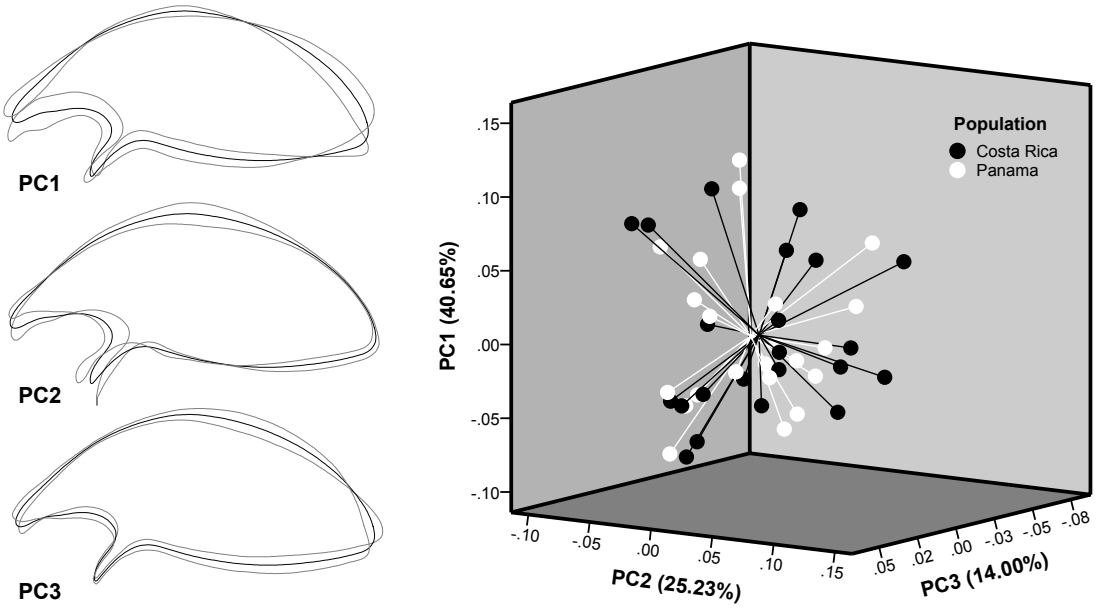


Figure 7. Male genital clasper shape. Left- Shape outlines corresponding to the mean (in black) and the standard deviations in both directions (in grey) are shown for the first three PCs that cumulatively account for nearly 80% of the total variation. Right- Plot of these axes showing little to no differentiation between populations.



3.4. COI gene fragment

A total of 12 males were extracted and amplified for the *COI* gene fragment. With the exception of one (CR1), all were successfully sequenced and analyzed. The neighbor joining tree (Kimura 2- parameter) depicts that all 11 sequences form a strongly supported monophyletic clade, together with a previously published sequence of *A. diversiformis* (Figure 8). No significant population clustering can be seen. Uncorrected pair-wise distances between the two populations were very small, ranging from 0.5 to 1.6%, and were even smaller within populations (CR: 0.3-0.8%; PAN: 0.1-1.4%; Table 3).

Figure 8. Neighbor joining tree of *COI* gene fragment in *A. diversiformis* (in bold; Costa Rica in dark grey; Panama in light grey). The letters and number after the species name is the code identifying the samples used in this study. Also included are closely related sequences recovered from GenBank (in black; with corresponding accession numbers). Bootstrap support values (for 1000 pseudo-replicates) higher than 50% are indicated at the branches.

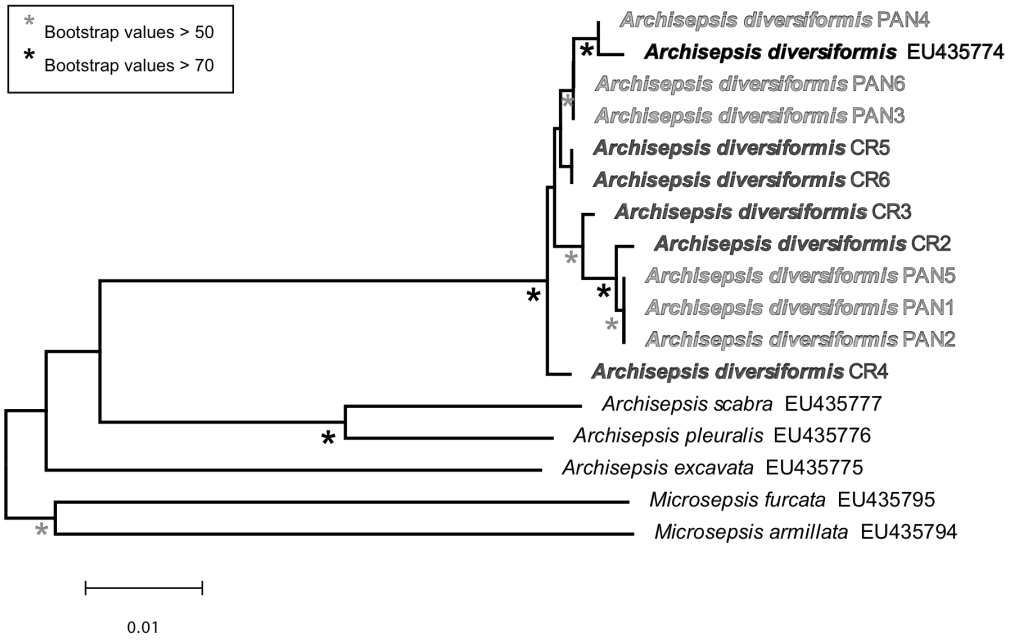


Table 3. Uncorrected pair-wise distances based on *COI* gene fragment between individuals from Costa Rica (CR) and Panama (PAN).

	CR2	CR3	CR4	CR5	CR6	PAN1	PAN3	PAN2	PAN4	PAN5	PAN6
Costa Rica 2	-	0.005	0.008	0.008	0.008	0.007	0.013	0.009	0.016	0.008	0.013
Costa Rica 3		-	0.005	0.003	0.003	0.007	0.008	0.009	0.010	0.008	0.008
Costa Rica 4			-	0.003	0.005	0.007	0.008	0.009	0.010	0.008	0.008
Costa Rica 5				-	0.003	0.007	0.005	0.009	0.008	0.008	0.005
Costa Rica 6					-	0.009	0.008	0.012	0.010	0.010	0.008
Panama 1						-	0.009	0.003	0.012	0.001	0.009
Panama 3							-	0.012	0.004	0.010	0.003
Panama 2								-	0.014	0.001	0.012
Panama 4									-	0.013	0.003
Panama 5										-	0.010
Panama 6											-

4. DISCUSSION

Here we studied the differentiation in reproductive behavior and morphology among Costa Rica (CR) and Panama (PAN) populations of the widespread neotropical fly *Archisepsis diversiformis*. Despite strong similarities in overall mating behavior, we demonstrate clear population differences, with behavioral elements that are specific to CR and PAN. Additionally, these populations exhibit some degree of pre-mating isolation in one-on-one mating trials that only resulted in copulations within but not across populations. However, there is no apparent post-mating incompatibility because both populations produced viable juveniles and adults after prolonged exclusive exposure to mating partners from the other population in group containers. Morphometric analysis of adult wings suggests that populations (and sexes) differ significantly in wing shape, whereas there is only moderate population differentiation in male fore femur shape and none in male genital clasper shape between CR and PAN. Based on divergence in *COI* sequences, which can be used to detect whether geographically distant populations of widespread species form undifferentiated clusters (Meier et al. 2006; Tan et al. 2010), we find that individuals from CR and PAN are genetically very similar, forming one strong monophyletic clade (Figure 8) and differing in pair-wise distances by less than 3%, which is the accepted (arbitrary) threshold for pair-wise distances in most closely related Dipteran species (Meier et al. 2006). Together, this evidence suggests that the significant differences in mating behavior and adult wing shape (a sexually monomorphic trait) between the two populations have arisen rather rapidly. More interestingly, they could be diverging faster than the sexually dimorphic male fore legs and genitalia. We suggest that both diversifying and stabilizing selection could operate differentially on these sexual and non-sexual traits at early stages of diversification in this widespread neotropical fly.

Mating behavior as mechanism of sexual isolation in A. diversiformis

In addition to identifying one behavioral element specific to this species, a mid leg display involving an angular extension of the mid tarsi (Figure 4A; clip 1), we document that males from PAN display a pre-copulatory mid leg 'swim' and females from CR a copulatory hind leg 'rub', both characters being absent in the other population (Table 1; clip 3, clip 5). Of particular interest is the potentially homologous behavior that is diverging between the two populations, involving the curling of the mid tarsi towards the female head (Figure 4B; clip 2). It is plausible that these differences are evolving due to direct selection on mate preference; or alternatively, this behavioral variation could have been enhanced by character displacement, especially if *A. diversiformis* from different populations across Central America interact occasionally. Character displacement specifically refers to the divergence of reproductive characters to avoid heterospecific

matings, particularly among sympatric siblings species (Crampton et al. 2011), and can be based on morphological, behavioral or even chemical traits (Friberg et al. 2008; Eltz et al. 2011; Bath et al. 2012).

In our one-on-one mating trials, both populations only showed matings within but not among populations. This appeared to be driven by both a male and female effect, with males mounting their hetero-population females less often, and possible female 'reluctance' evidenced by her shaking or 'kicking' behavior (clip 6). However, the latter behavior is also performed during con-population interactions, so it not possible to categorize it purely as a female response to hetero-population males. Despite such apparent pre-mating isolation, there was no post-mating incompatibility in producing viable F1 juveniles and adults, which is not surprising given that behavioral divergence in courtship and subsequent sexual isolation can evolve faster than hybrid inviability (Mendelson 2003; Van der Sluijs et al. 2008). We did not specifically address the fertility or overall viability of the F1 individuals in this study, and possible fitness detriments for these offspring remain to be investigated.

Population differentiation in wing shape

The dynamics of wing evolution in Diptera are often influenced by both natural and sexual selection (Hackman, 1964; Huey et al., 2000; Norry et al., 2001; Gidaszewski et al. 2009; Ribak et al. 2009; 2011). For instance, some *Drosophila* species are known to evolve wing morphology along altitudinal or latitudinal clines (Gilchrist et al., 2000; Santos et al., 2004; Routto et al. 2007). Another study on wing variation in an invasive moth in South America documents that high altitude individuals generally had larger but narrower wings than low altitude moths (Hernandez-L et al. 2010). We found similar morphological divergence in wing traits separating CR and PAN *A. diversiformis*. Both populations occur on different decomposing substrates and were sampled at different altitudes (CR on cattle dung at ~1000m, PAN mainly on monkey faeces at ~20m). Variation between the two populations was influenced strongly by changes in the internal landmarks of the wings and the edges of the wings, with the Panama population loading strongly in the positive direction indicated in Figure 5, implying smaller and wider wings. We suggest wing variation between CR and PAN could be attributed to natural selection shaping the wings to adjust to the local aerodynamic conditions, namely as a result of dissimilarities in flight requirements needed for locating resources in different environments (Norry et al. 2001; Hernandez-L et al. 2010).

Possible stabilizing selection on male fore femur and genital shape

Morphological diversity in sexual dimorphism and male genitalia has been well

documented across various animal groups (Eberhard 1985; Hosken and Stockley 2004). Many studies suggest that strong directional sexual selection (both pre- and post-copulatory) acting on these traits can lead to rapid population differentiation, and that these structures should be subject to continuous change (Arnqvist et al. 2000; Gavrilets 2000; Panhuis et al. 2001; Birkhead and Pizzari 2002; McPeek et al. 2008). However, other authors report that in many 'already-evolved', extant species it is more important to maintain species recognition, meaning that one should observe the effects of sustained stabilizing selection on male secondary sexual traits and genitalia within isolated populations, ultimately resulting in slow (or no) divergence in these structures (Bond et al. 2003; Simmons et al. 2009; Wojcieszek and Simmons 2012). Overall, we revealed strong individual variance for most femur and genital shape traits but moderate to no systematic differentiation between CR and PAN populations (Figure 6 and 7; Appendix C). It is possible that stabilizing selection is acting on the male fore femur and genital shape in *A. diversiformis*. Besides direct measurements of selection, one could test this by comparing divergence in these morphological structures to divergence in neural markers in a future Q_{ST}-F_{ST} study.

Overall we suggest that reproductive traits in these two populations are evolving at different evolutionary time scales and presumably under different selective forces. We highlight clear divergence in behavioral traits as well as moderate pre-mating isolation, implying that these characters are under directional sexual selection, whilst the population variation in wing shape could be a result of ecological selection perhaps related to altitude. However, contrary to the overwhelming evidence of rapid evolution in male sexually dimorphic structures, we present a possible case of stabilizing selection in male fore legs and genital claspers. We believe *A. diversiformis* represents an ideal system to further investigate patterns among evolving populations, especially along latitudinal or altitudinal clines.

5. REFERENCES

Arnqvist, G., M. Edvardsson, U. Friberg, and T. Nilsson. 2000. Sexual conflict promotes speciation in insects. Proceedings of the National Academy of Sciences 97:10460-10464.

Arnqvist, G., and L. Rowe. 2002. Antagonistic coevolution between the sexes in a group of insects. Nature 415:787-789.

Bath, E., N. Tatarnic, and R. Bonduriansky. 2012. Asymmetric reproductive isolation and interference in neriid flies: the roles of genital morphology and behaviour. Animal Behaviour 84:1331-1339.

Birkhead, T. R., and T. Pizzari. 2002. Postcopulatory sexual selection. Nature Reviews Genetics 3:262-273.

Blanckenhorn, W. U., A. F. G. Dixon, D. J. Fairbairn, M. W. Foellmer, P. Gibert, K. van der Linde, R. Meier, S. Nylin, S. Pitnick, C. Schoff, M. Signorelli, T. Teder, and C. Wiklund. 2007. Proximate causes of Rensch's rule: Does sexual size dimorphism in arthropods result from sex differences in development time? American Naturalist 169:245-257.

Bond, J. E., D. A. Beamer, M. C. Hedin, and P. Sierwald. 2003. Gradual evolution of male genitalia in a sibling species complex of millipedes (Diplopoda : Spirobolida : Rhinocricidae : Anadenobolus). Invertebrate Systematics 17:711-717.

Boul, K. E., W. C. Funk, C. R. Darst, D. C. Cannatella, and M. J. Ryan. 2007. Sexual selection drives speciation in an Amazonian frog. Proceedings of the Royal Society B-Biological Sciences 274:399-406.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Cure, C., N. Mathevon, R. Mundry, and T. Aubin. 2012. Acoustic cues used for species recognition can differ between sexes and sibling species: evidence in shearwaters. Animal Behaviour 84:239-250.

Dieckmann, U., and M. Doebeli. 1999. On the origin of species by sympatric speciation. Nature 400:354-357.

Dobzhansky, T., and E. Mayr. 1944. Experiments on sexual isolation in Drosophila I Geographic strains of Drosophila willistoni. Proceedings of the National Academy of Sciences of the United States of America 30:238-244.

Eberhard, W. G. 1985. Sexual Selection and Animal Genitalia. Eberhard, W. G. Sexual Selection and Animal Genitalia. Xii+244p. Harvard University Press: Cambridge, Mass., USA; London, England. Illus.

Eberhard, W. G. 2001. The functional morphology of species-specific clasping structures on the front legs of male Archisepsis and Palaeosepsis flies (Diptera, Sepsidae). Zoological Journal of the Linnean Society 133:335-368.

Eberhard, W. G. 2002. Physical restraint or stimulation? The function(s) of the modified front legs of male Archisepsis diversiformis (Diptera, Sepsidae). Journal of Insect Behavior 15:831-850.

Eberhard, W. G., and B. A. Huber. 1998. Copulation and sperm transfer in *Archisepsis* flies (Diptera, Sepsidae) and the evolution of their intromittent genitalia. Studia Dipterologica 5:217-248.

Eltz, T., F. Fritzsch, J. Ramirez Pech, Y. Zimmermann, S. R. Ramirez, J. J. G. Quezada-Euan, and B. Bembe. 2011. Characterization of the orchid bee Euglossa viridissima (Apidae: Euglossini) and a novel cryptic sibling species, by morphological, chemical, and genetic characters. Zoological Journal of the Linnean Society 163:1064-1076.

Emerson, S. B., and R. Ward. 1998. Male secondary sexual characteristics, sexual selection, and molecular divergence in fanged ranid frogs of Southeast Asia. Zoological Journal of the Linnean Society 122:537-553.

Fitzpatrick, M. J., and D. A. Gray. 2001. Divergence between the courtship songs of the field crickets *Gryllus texensis* and *Gryllus rubens* (Orthoptera, Gryllidae). Ethology 107:1075-1085.

Franz, N. M. 2003. Mating behaviour of Staminodeus vectoris (Coleoptera : Curculionidae), and the value of systematics in behavioural studies. Journal of Natural History 37:1727-1750.
Friberg, M., N. Vongvanich, A.-K. Borg-Karlson, D. J. Kemp, S. Merilaita, and C. Wiklund. 2008. Female mate choice determines reproductive isolation between sympatric butterflies. Behavioral Ecology and Sociobiology 62:873-886.
Gavrilets, S. 2000. Rapid evolution of reproductive barriers driven by sexual conflict. Nature 403:886-889.
Gleason, J. M., and M. G. Ritchie. 1998. Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex: Do sexual signals diverge the most quickly? Evolution 52:1493-1500.
Gray, D. A. 2005. Does courtship behavior contribute to species-level reproductive isolation in field crickets? Behavioral Ecology 16:201-206.
Gray, D. A., and W. H. Cade. 2000. Sexual selection and speciation in field crickets. Proceedings of the National Academy of Sciences of the United States of America 97:14449-14454.
Groning, J., and A. Hochkirch. 2008. Reproductive interference between animal species. Quarterly Review of Biology 83:257-282.
Hammer, Ø., D. A. T. Harper, and P. D. Ryan. 2001. PAST: Paleontological statistics software package for education and data analysis. Pp. 9. Palaeontologia Electronica.
Hernandez-L, N., A. R. Barragan, S. Dupas, J. F. Silvain, and O. Dangles. 2010. Wing shape variations in an invasive moth are related to sexual dimorphism and altitude. Bulletin of Entomological Research 100:529-541.
Hoffmann, A. A., and J. Shirriffs. 2002. Geographic variation for wing shape in Drosophila serrata. Evolution 56:1068-1073.
Hosken, D. J., and P. Stockley. 2004. Sexual selection and genital evolution. Trends in Ecology & Evolution 19:87-93.
Ingram, K. K., T. Laamanen, N. Puniamoorthy, and R. Meier. 2008. Lack of morphlogical coevolution between male forelegs and female wings in *Themira* (Sepsidae: Dipera: Insecta). Biological Journal of the Linnean Society 93:227-238.
Iwata, H., and Y. Ukai. 2002. SHAPE: A computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. Journal of Heredity 93:384-385.
Kim, Y.-K., M. Ruiz-Garcia, D. Alvarez, D. R. Phillips, and W. W. Anderson. 2012. Sexual isolation between North American and Bogota strains of Drosophila pseudoobscura. Behavior Genetics 42:472-482.
Klappert, K., D. Mazzi, A. Hoikkala, and M. G. Ritchie. 2007. Male courtship song and female preference variation between phylogeographically distinct populations of Drosophila montana. Evolution 61:1481-1488.
Kraaijeveld, K., F. J. L. Kraaijeveld-Smit, and M. E. Maan. 2011. Sexual selection and speciation: the comparative evidence revisited. Biological Reviews 86:367-377.
Kuhl, F. P., and C. R. Giardina. 1982. Elliptic fourier features of a closed contour. Computer Graphics and Image Processing 18:236-258.
Luan, J.-B., P. J. De Barro, Y.-M. Ruan, and S.-S. Liu. 2013. Distinct behavioural strategies underlying asymmetric mating interactions between invasive and indigenous whiteflies. Entomologia Experimentalis Et Applicata 146:186-194.
McPeek, M. A., L. Shen, J. Z. Torrey, and H. Farid. 2008. The tempo and mode of three-dimensional morphological evolution in male reproductive structures. American Naturalist 171:E158-E178.
Meier, R., S. Kwong, G. Vaidya, and P. K. L. Ng. 2006. DNA Barcoding and Taxonomy in Diptera: a Tale of High Intraspecific Variability and Low Identification Success. Systematic Biology 55:715-728.
Mendelson, T. C. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae : Etheostoma). Evolution 57:317-327.

Norry, F. M., O. A. Bubliy, and V. Loeschcke. 2001. Developmental time, body size and wing loading in Drosophila buzzatii from lowland and highland populations in Argentina. Hereditas 135:35-40.
Ozerov, A. L. 2005. World catalogue of the family Sepsidae (Insecta: Diptera). Zoologicheskie issledovania (Zoological Studies) 8:1-74.
Panhuis, T. M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. Trends in Ecology & Evolution 16:364-371.
Peretti, A. V., and A. Cordoba-Aguilar. 2007. On the value of fine-scaled behavioural observations for studies of sexual coercion. Ethology Ecology & Evolution 19:77-86.
Pinto, J. D. 1977. Comparative sexual-behavior in blister beetles of subtribe Eupomphina (Coleoptera-Meloidae), and an evaluation of its taxonomic significance. Annals of the Entomological Society of America 70:937-951.
Podos, J., S. K. Huber, and B. Taft. 2004. Bird song: the interface of evolution and mechanism. . Annual Review of Ecology, Evolution, and Systematics 35.
Podos, J., and P. S. Warren. 2007. The evolution of geographic variation in birdsong. Pp. 403-458. Advances in the Study of Behavior, Vol 37.
Prohl, H., S. Hagemann, J. Karsch, and G. Hobel. 2007. Geographic variation in male sexual signals in strawberry poison frogs (Dendrobates pumilio). Ethology 113:825-837.
Puniamoorthy, N., W. U. Blanckenhorn, and M. A. Schäfer. 2012a. Differential investment in pre- versus post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae). Journal of Evolutionary Biology 25:2253–2263.
Puniamoorthy, N., M. R. B. Ismail, D. S. H. Tan, and R. Meier. 2009. From kissing to belly stridulation: comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behaviour of 27 species of sepsid flies (Diptera: Sepsidae). Journal of Evolutionary Biology 22:2146-2156.
Puniamoorthy, N., M. A. Schäfer, and W. U. Blanckenhorn. 2012b. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). Evolution 66:2117–2126.
Puniamoorthy, N., K. F. Y. Su, and R. Meier. 2008. Bending for love: losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae : Diptera). Bmc Evolutionary Biology 8.
Rohlf, F. J. 2006. TpsDig. Department of Ecology and Evolution, State University of New York., New York.
Routto, J., D. Mazzi, K. Van Der Linde, P. Mirol, R. K. Butlin, and A. Hoikakala. 2007. The extent of variation in male song, wing and genital characters among allopatric Drosophila montana populations. Journal of Evolutionary Biology 20:1591-1601.
Schutze, M. K., D. K. Yeates, G. C. Graham, and G. Dodson. 2007. Phylogenetic relationships of antlered flies, *Phytalmia* Gerstaecker (Diptera: Tephritidae): the evolution of antler shape and mating behaviour. Australian Journal of Entomology 46:281-293.
Seehausen, O., J. J. M. vanAlphen, and F. Witte. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. Science 277:1808-1811.
Simmons, L. W., C. M. House, J. Hunt, and F. Garcia-Gonzalez. 2009. Evolutionary Response to Sexual Selection in Male Genital Morphology. Current Biology 19:1442-1446.
Simmons, L. W., M. Zuk, and J. T. Rotenberry. 2001. Geographic variation in female preference functions and male songs of the field cricket Teleogryllus oceanicus. Evolution 55:1386-1394.
Snook, R., A. Robertson, H. S. Crudgington, and M. G. Ritchie. 2005. Experimental manipulation of sexual selection and the evolution of courtship song in *Drosophila pseudoobsura*. Behavior Genetics 35:245-255.
Tan, D., Y. Ang, G. Lim, M. Ibrahim, and R. Meier. 2010. From 'cryptic species' to integrative taxonomy: an iterative pro sequences, morphology, and behaviour

leads to the resurrection of *Sepsis pyrrhosoma* (Sepsidae: Diptera). Zoologica Scripta.
Tan, D. S. H., S. R. Ng, and R. Meier. 2011. New information on the evolution of mating behaviour in Sepsidae (Diptera) and the cost of male copulations in *Saltella sphondylii*. Organisms, Diversity and Evolution 11:253-261.
Tamura , K., Dudley, J., Nei, M. and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular biology and evolution. 24:1596–1599.
Van der Sluijs, I., T. J. M. Van Dooren, O. Seehausen, and J. J. M. Van Alphen. 2008. A test of fitness consequences of hybridization in sibling species of Lake Victoria cichlid fish. Journal of Evolutionary Biology 21:480-491.
Vedenina, V. Y., N. K. Kulygina, and A. K. Panyutin. 2007. Isolation mechanisms in the closely related grasshopper species, Chorthippus albomarginatus and Ch. oschei (Orthoptera, Acrididae). Zoologichesky Zhurnal 86:537-546.
Williams, T. H., and T. C. Mendelson. 2010. Behavioral Isolation Based on Visual Signals in a Sympatric Pair of Darter Species. Ethology 116:1038-1049.
Wojcieszek, J. M., and L. W. Simmons. 2012. Evidence for stabilizing selection and slow divergent evolution of male genitalia in a millipede (*Antichirpus variabilis*). Evolution 66:1138-1153.

APPENDIX A

Character matrix for *Archisepsis diversiformis* within-population behavior trials ('-' : not applicable)

Population	Trial ID#	Behavior characters																														Copulation duration
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
Costa Rica	6	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	34.42
Costa Rica	7	0	0	0	0	2	0	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	25.32
Costa Rica	8	0	0	0	1	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	34.37
Costa Rica	10	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	28.73
Costa Rica	15	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	32.28
Costa Rica	16	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	24.00
Costa Rica	17	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	23.18
Costa Rica	18	0	0	0	0	2	0	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	26.68
Costa Rica	19	0	0	0	0	2	0	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	23.78
Costa Rica	20	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	30.83
Costa Rica	21	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	25.27
Costa Rica	23	0	0	0	0	2	0	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	31.52
Costa Rica	24	0	0	0	0	2	0	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	22.42
Costa Rica	25	0	0	0	0	2	1	1	1&3	0	1	-	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	38.28
Panama	1	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	39.75
Panama	2	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	29.00
Panama	7	0	0	0	0	2	0	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	26.03
Panama	8	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	26.42
Panama	10	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	0	2	0	0	0	1	1	0	0	2	-	0	1	0	0	2	0	32.58
Panama	11	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	34.67
Panama	14	0	0	0	0	2	0	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	28.50
Panama	16	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	26.53
Panama	18	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	27.00
Panama	21	0	0	0	0	2	0	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	23.07
Panama	22	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	22.05
Panama	23	0	0	0	0	2	1	1	1&3	1	1&2	0	2	0	0	0	1	2	0	0	0	1	1	0	0	2	-	0	1	0	0	0	0	27.93

Character descriptions modified from Puniamoorthy et al. (2009) & Tan et al. (2011). New character states are in **bold**.

(1) 'Circling'—0: Absent (Male approach without gliding motion); 1: Present (Male circling female in a gliding motion, head and thorax leading the change of direction with abdomen bent at an angle)

(2) Initial mount—0: Male jumps or climbs onto female; 1: Male bends abdomen anteriad to establish direct genital contact

(3) Effect of struggle on in-copula position of pair—0: Females do not flip over; 1: Pair flipping over resulting in male on his back and female with her legs in air)

(4) Male proboscis–female interaction—0: No contact between male proboscis and female; 1: Male extends proboscis to 'kiss' female ocelli; 2: Male extends proboscis to tap dorsal part of female thorax

(5) Male grasp of female wingbase—0: Forelegs resting on female thorax; 1: Forelegs release wingbase only at separation; 2: Forelegs release female wings well before separation

(6) Male foreleg after release—0: Resting against female thorax (no movement); 1: Male foreleg interacting with female thorax and, or foreleg

(7) Midleg position—0: Male midlegs always in contact with female; 1: Male mid legs are stretched out away from female body i.e. 'balancing' (for extended period); 2: Male midlegs are not stretched out and are not in contact with female

(8) Motion restricted to mid tarsus—0: No independent tarsal movements; 1: Curling (movement of the tarsi 2–4 against the barsitarsus); 2: Quiver (vibration of entire tarsus without movement of tibia or femur); **3: Display (extension of entire tarsus at an angle away from the female head without vibration)**

(9) Non-contact midleg movement—0: Simultaneous usage of both midlegs; 1: Simultaneous and alternate; 2: Alternate usage of both midlegs

(10) Direction of midleg movement—0: Male midleg stretched out and stationary; 1: Male midleg towards female eye; 2: Male midlegs move posteriad

(11) Male midleg movement away from female head—0: Smooth return without any interruptions; 1: Return interrupted by midleg waves

(12) Midleg rotation during tarsal curl—0: Midleg curling in a horizontal plane; 1: Curling direction shifting from horizontal to vertical plane through leg rotation; 2: Curling in a vertical plane

(13) Number of tarsal curls per midleg movement—0:nSingle curl per midleg movement; 1: Multiple curls

(14) Midleg interaction with female head—0: No with contact female head; 1: Male uses his mid tarsi to rub head; 2: Male uses his mid tarsi to tap head (singular movements); 3: Male uses midleg to 'beat' female head

(15) Midleg interaction with female abdomen—0: No with contact female abdomen; 1: Male uses his midlegs to tap female abdomen

(16) Midleg interaction with female thorax—0: Absent; 1: Midlegs tap lateral surface (singular movements); 2: Midlegs 'stroke' lateral surface (extended rubbing); 3: Midlegs 'stroke' dorsal surface (extended rubbing)

(17) Midleg interaction with female wings—0: Male midlegs rest on female wings; 1: Male

midlegs used to forcibly bend down female wings; **2: Male midlegs used to rub female wings**

(18) Midleg to midleg grasp—0: Male midlegs not holding female mid legs; 1: Male uses midlegs to hold onto female midlegs

(19) Contact of male hindleg with substrate—0: Hindleg not in contact with substrate when female moves; 1: Mounted male's hindleg is dragged along substrate when female moves; 2: Mounted male walks in tandem with female instead of being dragged

(20) Non-contact movement of male hindlegs—0: Absent; 1: Hindlegs curl backwards 360° like a backward 'butterfly stroke'; 2: 'Cycling' (i.e. like a peddling motion); 3: Curling (Hindlegs stretched out and vibration of the 2–4 tarsal segments against the barsitarsus)

(21) Usage of hindlegs—0: No direct contact with female; 1: Tap ventral side of female abdomen; 2: Rub repeatedly on dorsal side of female wing; 3: Rub repeatedly wing margin

(22) Midleg–hindleg rub—0: No contact between male mid and hindlegs; 1: Males rub their hindlegs with their midlegs

(23) Part of female body male rubs after rubbing his hindlegs—0: Wing; 1: Thorax; 2: Head; 3: Abdomen; 4: Midlegs

(24) Movement of male abdomen—0: Abdomen main- tained horizontally without any movement; 1: Abdo- men lifted >90° over the thorax when mounted on female; 2: Male shakes abdomen vigorously from side to side (prior to mounting)

(25) Surstylus stimulation prior to genital contact—0: Males only lower abdomen to establish genital contact; 1: Male repeatedly lowers abdomen to stimulate female using the surstylis, either by vibration or by tapping on dorsal surface of female abdomen; 2: Male repeatedly lowers surstylus to stimulate female close to her genital opening; 3: Male lowers surstylus to stimulate on ventral surface of female abdomen

(26) Male tapping female with modified fourth sternites— 0: Modified 4th sternites of males used to tap or stroke dorsal part of female abdomen; 1: Ventral part of female abdomen

(27) Separation after copulation—0: Quick (one or two 180° turns by the male); 1: Long (involving a prolonged struggle between male and female in trying to break genital contact); 2: Quick but not involving turns by the male

(28) Female Shake—0: Absent (no violent side to side movement); 1: Present

(29) Type of female shake—0: Horizontally; 1: Vertically

(30) Female foreleg movements—0: No significant move- ments of forelegs; 1: Female intermittently lifts forelegs off the substrate; 2: Female repeatedly lifts forelegs above head

(31) Female hindleg movements—0: Female hindlegs not used to interact with male; 1: Female hindleg used to 'kick' male; 2: Female hindleg 'rubbing' male hindlegs

(32) Female ejection of ovipositor when male is mounted—0: Absent; 1: Female ejects ovipositor when after genital contact; 2: Female ejects ovipositor prior to genital contact

APPENDIX B

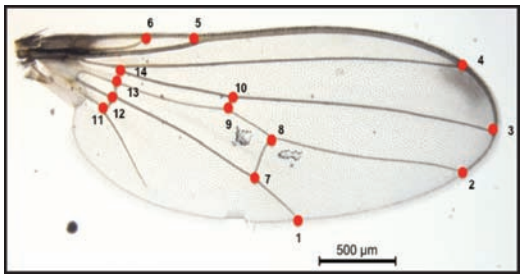
Heterospecific Trial #	Outcome (0: No copulation| 1: Copulation)	Focal ♀ ID	♂ ID	Description
Costa Rica females with Panama males				
1	0	CR ♀ A	PAN ♂ A	No attempts
2	0	CR ♀ B	PAN ♂ B	No attempts
3	0	CR ♀ C	PAN ♂ B	Mount but no precop
4	0	CR ♀ D	PAN ♂ D	No attempts
5	0	CR ♀ E	PAN ♂ E	No attempts
6	0	CR ♀ F	PAN ♂ F	No attempts
7	0	CR ♀ G	PAN ♂ G	No attempts
8	0	CR ♀ H	PAN ♂ H	Mount but no precop
9	0	CR ♀ I	PAN ♂ I	Mount but no precop
10	0	CR ♀ J	PAN ♂ J	Mount but no precop
11	0	CR ♀ K	PAN ♂ K	Few attempts
12	0	CR ♀ L	PAN ♂ L	No attempts
13	0	CR ♀ M	PAN ♂ M	No attempts
14	0	CR ♀ N	PAN ♂ N	No attempts
15	0	CR ♀ O	PAN ♂ O	No attempts
16	0	CR ♀ P	PAN ♂ P	No attempts
Panama females with Costa Rica males				
1	0	BCI ♀ A	SA ♂ A	Several mounting and quick dismount
2	0	BCI ♀ A	SA ♂ B	Few attempts
3	0	BCI ♀ B	SA ♂ C	No attempts
4	0	BCI ♀ C	SA ♂ D	Immediate mount but no precop
5	0	BCI ♀ D	SA ♂ E	No attempts
6	0	BCI ♀ E	SA ♂ F	No attempts
7	0	BCI ♀ F	SA ♀ F	No attempts
8	0	BCI ♀ G	SA ♂ G	No attempts
9	0	BCI ♀ H	SA ♂ H	No attempts
10	0	BCI ♀ I	SA ♂ I	Few attempts
11	0	BCI ♀ J	SA ♂ J	One attempt
12	0	BCI ♀ K	SA ♂ K	Few approaches but no attempts
13	0	BCI ♀ L	SA ♂ L	No attempts
14	0	BCI ♀ M	SA ♂ M	Prolonged precop attempt
15	0	BCI ♀ N	SA ♂ N	No attempts
16	0	BCI ♀ O	SA ♂ O	Multiple attempts
17	0	BCI ♀ P	SA ♂ P	No attempts

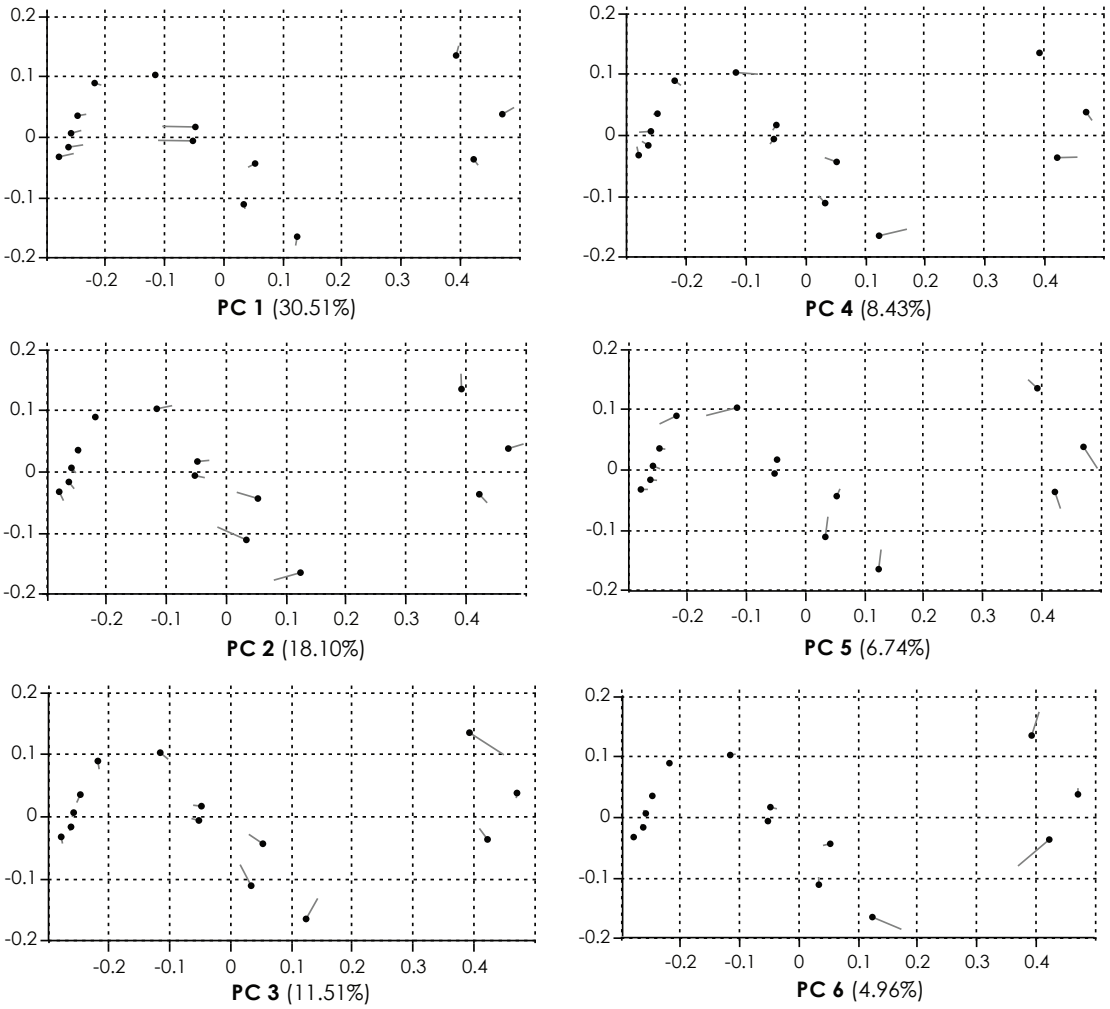
Conspecific Trial #	Outcome (0: No copulation| 1: Copulation)	Focal ♀ ID	♂ ID	Description
Panama females with Panama males				
1	1	BCI ♀ **A**	BCI ♂ **A**	Immediate attempt and copulation
2	0	BCI ♀ **B**	BCI ♂ **B**	Prolonged precop behavior but no copulation
3	1	BCI ♀ **C**	BCI ♂ C	Copulation
4	1	BCI ♀ D	BCI ♂ D	Copulation
5	0	BCI ♀ E	BCI ♂ E	Attempts but no copulation
6	0	BCI ♀ F	BCI ♂ F	Attempts but no copulation
7	1	BCI ♀ G	BCI ♂ G	Copulation
8	1	BCI ♀ H	BCI ♂ H	Immediate attempt and copulation
9	0	BCI ♀ I	BCI ♂ I	Prolonged precop and multiple mounts
10	1	BCI ♀ J	BCI ♂ J	Copulation
11	0	BCI ♀ K	BCI ♂ K	Prolonged precop and mounts
12	1	BCI ♀ L	BCI ♂ L	Copulation
13	0	BCI ♀ M	BCI ♂ M	Attempts but no copulation
14	0	BCI ♀ N	BCI ♂ N	Attempts but no copulation
15	1	BCI ♀ O	BCI ♂ O	Many mounts and copulation
16	0	BCI ♀ P	BCI ♂ P	Attempts but no copulation
Costa Rica females with Costa Rica males				
1	1	SA ♀ **A**	SA ♂ **A**	Copulation
2	0	SA ♀ **B**	SA ♂ **B**	Attempts but no copulation
3	0	SA ♀ **C**	SA ♂ **C**	Prolonged precops
4	0	SA ♀ D	SA ♂ **D**	Prolonged precops
5	1	SA ♀ E	SA ♂ E	Copulation
6	0	SA ♀ F	SA ♂ F	No attempts
7	0	SA ♀ G	SA ♂ G	Prolonged precops
8	0	SA ♀ H	SA ♂ H	Attempts but no copulation
9	1	SA ♀ I	SA ♂ I	Copulation
10	1	SA ♀ J	SA ♂ J	Cop at 5' end at 35'41
11	0	SA ♀ K	SA ♂ K	Attempts but no copulation
12	0	SA ♀ L	SA ♂ L	Attempts but no copulation
13	0	SA ♀ M	SA ♂ M	Attempts but no copulation
14	0	SA ♀ N	SA ♂ N	No attempts
15	1	SA ♀ O	SA ♂ O	Copulation
16	1	SA ♀ P	SA ♂ P	Copulation

APPENDIX C

Adult wing shape

- 14 wing landmark displacements for the first 6 PCs; (+ 0.1 vector displacement of PC) from the overall mean Procrustes shape.





Significant differences between populations: PC 1, 3 & 6
Significant differences between sexes: PC 2, 4, 5 & 6.

- Percentage variation explained by 15 PCs

	% variation explained	
	Individual PC	Cumulative
PC1	30.512	30.512
PC2	18.095	48.607
PC3	11.51	60.117
PC4	8.4288	68.5458
PC5	6.7447	75.2905
PC6	4.9598	80.2503

- Results of MANOVA based on 11 PCs

Multivariate Tests

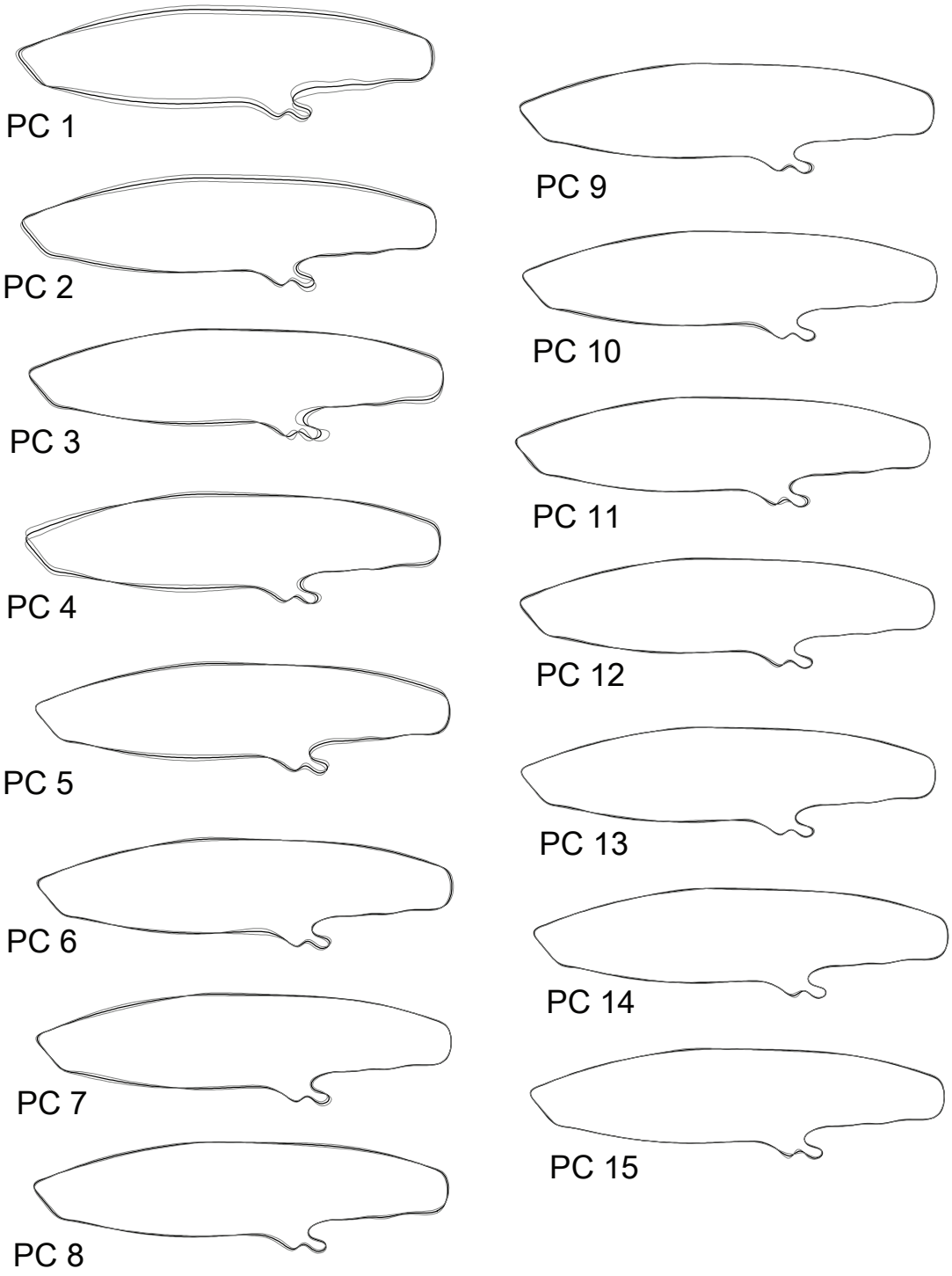
Effect		Value	F	Hypothesis df	Error df	Sig.
Population	Wilks' Lambda	.549	19.052a	6.000	139.000	.000
Sex		.468	26.335a	6.000	139.000	.000
Population*Sex		.961	.940a	6.000	139.000	.468

ANOVA of individual PCs

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Population	PC1	27.732	1	27.732	35.177	.000
	PC2	.354	1	.354	.363	.548
	PC3	25.490	1	25.490	31.062	.000
	PC4	.599	1	.599	.617	.433
	PC5	.817	1	.817	1.299	.256
	PC6	5.938	1	5.938	6.407	.012
Sex	PC1	1.897	1	1.897	2.406	.123
	PC2	5.440	1	5.440	5.574	.020
	PC3	.393	1	.393	.479	.490
	PC4	6.535	1	6.535	6.736	.010
	PC5	54.308	1	54.308	86.359	.000
	PC6	6.589	1	6.589	7.108	.009
Population*Sex	PC1	.404	1	.404	.512	.475
	PC2	1.222	1	1.222	1.253	.265
	PC3	.169	1	.169	.205	.651
	PC4	.061	1	.061	.063	.802
	PC5	.390	1	.390	.620	.432
	PC6	3.425	1	3.425	3.696	.057
Error	PC1	113.526	144	.788		
	PC2	140.536	144	.976		
	PC3	118.171	144	.821		
	PC4	139.708	144	.970		
	PC5	90.557	144	.629		
	PC6	133.477	144	.927		

Male fore femur shape

- Shape outlines with mean and standard deviations in both directions for each individual PC.



- Percentage variation explained by 15 PCs

	% variation explained	
	Individual PC	Cumulative
PC1	33.3114	33.3114
PC2	18.4585	51.7699
PC3	12.7503	64.5202
PC4	9.7377	74.2579
PC5	5.1193	79.3772
PC6	2.8959	82.2731
PC7	2.6654	84.9386
PC8	2.2844	87.223
PC9	1.9545	89.1775
PC10	1.3252	90.5027
PC11	1.0973	91.6
PC12	0.8199	92.4199
PC13	0.6775	93.0974
PC14	0.6516	93.749
PC15	0.5863	94.3353

- Results of MANOVA based on 15 PCs

Multivariate Tests

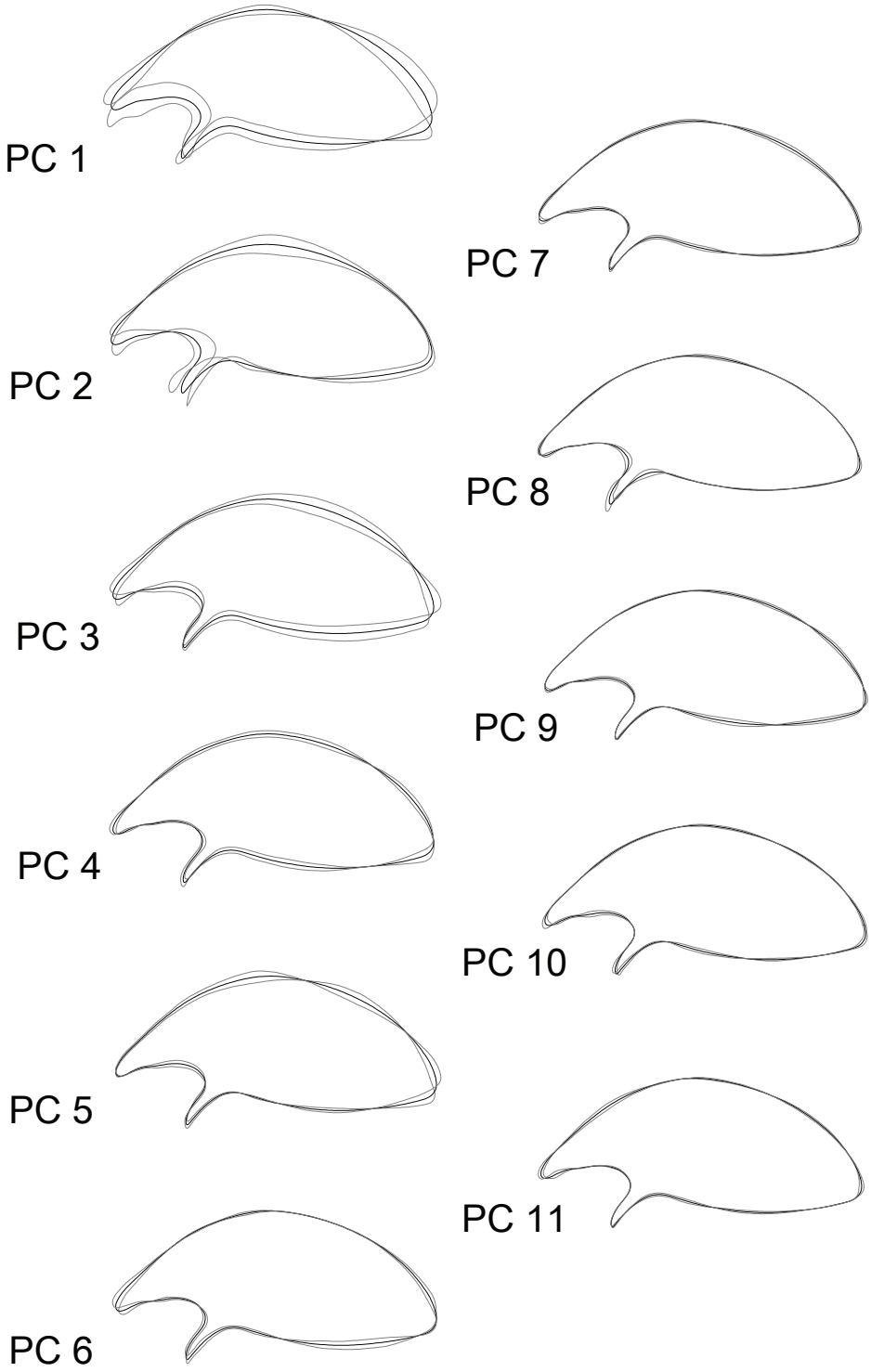
Effect		Value	F	Hypothesis df	Error df	Sig.
Population	Wilks' Lambda	.577	6.365a	15.000	130.000	.000

ANOVA of individual PCs

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Population	PC1	.000	1	.000	.700	.404
	PC2	.001	1	.001	3.188	.076
	PC3	.000	1	.000	1.224	.270
	PC4	.001	1	.001	8.674	.004
	PC5	.001	1	.001	28.045	.000
	PC6	.000	1	.000	8.201	.005
	PC7	.000	1	.000	.727	.395
	PC8	.000	1	.000	2.182	.142
	PC9	.000	1	.000	.374	.542
	PC10	.000	1	.000	5.058	.026
	PC11	.000	1	.000	3.829	.052
	PC12	.000	1	.000	3.518	.063
	PC13	.000	1	.000	.699	.404
	PC14	.000	1	.000	.530	.468
	PC15	.000	1	.000	.013	.910
Error	PC1	.047	144	.000		
	PC2	.025	144	.000		
	PC3	.018	144	.000		
	PC4	.013	144	.000		
	PC5	.006	144	.000		
	PC6	.004	144	.000		
	PC7	.004	144	.000		
	PC8	.003	144	.000		
	PC9	.003	144	.000		
	PC10	.002	144	.000		
	PC11	.002	144	.000		
	PC12	.001	144	.000		
	PC13	.001	144	.000		
	PC14	.001	144	.000		
	PC15	.001	144	.000		

Male clasper shape

- Shape outlines with mean and standard deviations in both directions for each individual PC.



- Percentage variation explained by 11 PCs

	% variation explained	
	Individual PC	Cumulative
PC1	40.6531	40.6531
PC2	25.2311	65.8843
PC3	13.988	79.8722
PC4	5.2374	85.1096
PC5	4.1083	89.2179
PC6	2.3233	91.5412
PC7	1.5416	93.0828
PC8	1.072	94.1548
PC9	0.9544	95.1093
PC10	0.8464	95.9557
PC11	0.6714	96.627

- Results of MANOVA based on 11 PCs

Multivariate Tests

Effect		Value	F	Hypothesis df	Error df	Sig.
Population	Wilks' Lambda	.668	1.491	11.000	33.000	.182

ANOVA of individual PCs

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Population	PC1	.004	1	.004	1.628	.209
	PC2	.004	1	.004	2.783	.103
	PC3	.000	1	.000	.142	.708
	PC4	.000	1	.000	.673	.417
	PC5	.000	1	.000	1.843	.182
	PC6	.001	1	.001	7.320	.010
	PC7	.000	1	.000	.368	.547
	PC8	.000	1	.000	.052	.822
	PC9	.000	1	.000	.339	.563
	PC10	.000	1	.000	.280	.599
	PC11	.000	1	.000	.237	.629
Error	PC1	.113	43	.003		
	PC2	.069	43	.002		
	PC3	.040	43	.001		
	PC4	.015	43	.000		
	PC5	.011	43	.000		
	PC6	.006	43	.000		
	PC7	.004	43	.000		
	PC8	.003	43	.000		
	PC9	.003	43	.000		
	PC10	.002	43	.000		
	PC11	.002	43	.000		

CONCLUSIONS & FUTURE DIRECTIONS

The research presented in this dissertation reiterates the usefulness of conducting extensive within species studies including different types of data (morphological, behavioral and molecular) to study the effects of selection and diversification among widespread species.

Chapter one showed that sexual selection on male body size accounts for the geographic reversal of SSD in the widespread dung fly *Sepsis punctum.* A combined approach including common garden and fitness experiments in the laboratory established that SSD was male-biased in Europe and female-biased in North America. The intensity of sexual selection increased with male body size and operational sex ratio in the Europe and was significantly stronger than North America. Although there was fecundity selection on female body size, it did not differ between the continents. Finally, viability selection on intrinsic adult lifespan in the laboratory was overall nil. This chapter confirmed the differential equilibrium model of SSD whereby differences in sexual selection intensity account for the reversal in SSD between North America and Europe (Puniamoorthy et al. 2012a). **Chapter two** documented that flies from both continents differed with respect to their investment in pre- versus post-copulatory traits. European populations exhibited higher re-mating rates and males have consequently evolved relatively larger testes exhibiting steeper hyper-allometry with body size. In sharp contrast, North American populations showed increased investment in mate acquisition prior to copulation, with more mounting attempts and a distinctive abdominal courtship display that was completely absent in Europe. Chapter two also documented an east-west gradient in the intensity of the display in North America. Overall, this study suggested a trade-off between traits that enhance mate acquisition and those enhancing fertilization success (Puniamoorthy et al. 2012b).

Chapter three investigated the underlying population genetic structure in this species and demonstrated a clear differentiation between the continents as well as strong isolation-by-distance within the continents. Based on six microsatellite markers and the *COI* gene fragment, *S. punctum* populations exhibit global and continental differences in genetic variation among the independently inherited molecular data comparable to or even higher than that for some other widespread Dipteran species (Caracristi and Schlotterer 2003; Routto et al. 2007; Puslednik et al. 2012). Considering the variation in

morphological and behavioral traits documented in Chapters one and two, these patterns of genetic differentiation are indicative of incipient speciation in *S. punctum*. Studies in other insect groups indicate that chemical cues can diverge strongly during incipient speciation (Caceres et al. 2009; Symonds et al. 2009). As such, **chapter four** explored volatile organic compounds in *S. punctum* and two other outgroup sepsid species. Out of 29 compounds identified overall, 9 were compounds specific to *S. punctum,* which could be associated with a glandular male organ involved in copulatory courtship. This chapter also highlighted differences in VOCs between the continents, though further work is required to address their functional significance.

The final **chapter five** documented that mating behavior evolves faster than sexually dimorphic structures in another widespread sepsid fly, *Archisepsis diversiformis*. Certain behavioral elements performed during mating were clearly population-specific, and some pre-mating isolation was apparent, although viable F1 population hybrids were produced. Furthermore, morphometric analysis indicates that the populations differed significantly in wing shape but only moderately in male fore femur shape and not at all in male genital clasper shape, and populations were genetically highly similar with pairwise distances of less than 1.6%. Thus it appears that differences between these two populations have arisen rather rapidly, with behavior diverging faster than morphology, presumably mediated by both directional and stabilizing selection on sexual and non-sexual traits at early stages of diversification in this neotropical fly.

Future directions

The work presented in this dissertation has created new avenues for future research. For instance, the higher re-mating rates in females accompanied by an increased investment in male testes observed in European *S. punctum* populations suggest that mechanisms of post-copulatory sexual selection, particularly sperm competition, are at play. The divergence in sperm morphology among insects (Pitnick et al. 2009; Higginson et al. 2012) has been well documented, and there is a wealth of information suggesting that ejaculate production and investment in sperm form represents a significant cost to males (Pitnick 1996; Baer et al. 2006; Ferkau and Fischer 2006; Del Castillo and Gwynne 2007; Engqvist 2011; Lupold et al. 2011). Many insect species are subject to high levels of selection via sperm competition (Lorch et al. 1993; Simmons et al. 1999; Andres and Rivera 2000), because females of most species mate multiply and possess organs specialized for long term sperm storage, thus facilitating the co-occurrence of sperm from several males within the female reproductive tract during fertilization (Parker 1970). So far, little is known about post-copulatory sexual selection in sepsid species. Preliminary data on male sperm investment suggest that patterns of sperm precedence

might be quite variable among species, from mixed paternity to strong last male biased paternity (Schultz 1999; Martin and Hosken 2002). Clearly, the post-copulatory determinants of male-female reproductive interactions in sepsid flies and especially *S. punctum* require further research, which I aim to pursue in a future post-doc.

Phenotypic differentiation across a species range can result from diversifying selection or genetic drift, or the combination of both. Theory predicts that divergent selection can result in differentiation in phenotypic traits (Q_{ST}) that is greater than the divergence in neutral genetic markers (F_{ST}). On the other hand, stabilizing selection is expected to reduce phenotypic variation across environments, so the opposite pattern is expected ($Q_{ST} < F_{ST}$). The null model hypothesizes that quantitative traits evolve neutrally due to drift ($Q_{ST} = F_{ST}$) (Demont et al. 2008; Wojcieszek and Simmons 2012). The distinct variation in wing morphology and mating behavior between the Costa Rica and Panama populations in *A. diversiformis* could indicate that gene flow is insufficient to override population specific selection on these traits even at a relatively small geographical scale. Hence, given the widespread range distribution of this species, a Q_{ST}-F_{ST} approach is highly promising to differentiate between selection and drift affecting reproductive traits. Again, this is an avenue that I intend to pursue in the future.

REFERENCES

Andres, J. A., and A. C. Rivera. 2000. Copulation duration and fertilization success in a damselfly: an example of cryptic female choice? Animal Behaviour 59:695-703.

Baer, B., S. A. O. Armitage, and J. J. Boomsma. 2006. Sperm storage induces an immunity cost in ants. 441:872-875.

Caceres, C., D. F. Segura, M. T. Vera, V. Wornoayporn, J. L. Cladera, P. Teal, P. Sapountzis, K. Bourtzis, A. Zacharopoulou, and A. S. Robinson. 2009. Incipient speciation revealed in Anastrepha fraterculus (Diptera; Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. Biological Journal of the Linnean Society 97:152-165.

Caracristi, G., and C. Schlotterer. 2003. Genetic differentiation between American and European Drosophila melanogaster populations could be attributed to admixture of African alleles. Molecular Biology and Evolution 20:792-799.

Del Castillo, R. C., and D. T. Gwynne. 2007. Increase in song frequency decreases spermatophore size: correlative evidence of a macroevolutionary trade-off in katydids (Orthoptera : Tettigoniidae). Journal of Evolutionary Biology 20:1028-1036.

Demont, M., W. U. Blanckenhorn, D. J. Hosken, and T. W. J. Garner. 2008. Molecular and quantitative genetic differentiation across Europe in yellow dung flies. Journal of Evolutionary Biology 21:1492-1503.

Engqvist, L. 2011. Male attractiveness is negatively genetically associated with investment in copulations. Behavioral Ecology 22:345-349.

Ferkau, C., and K. Fischer. 2006. Costs of reproduction in male Bicyclus anynana and Pieris napi butterflies: Effects of mating history and food limitation. Ethology 112:1117-1127.

Higginson, D. M., K. B. Miller, K. A. Segraves, and S. Pitnick. 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proceedings of the National Academy of Sciences of the United States of America 109:4538-4543.

Lorch, P. D., G. S. Wilkinson, and P. R. Reillo. 1993. Copulation duration and sperm precedence in the stalk-eyed fly Cyrtodiopsis whitei (Diptera: Diopsidae). Behavioral Ecology and Sociobiology 32:303-311.

Lupold, S., M. K. Manier, O. Ala-Honkola, J. M. Belote, and S. Pitnick. 2011. Male Drosophila melanogaster adjust ejaculate size based on female mating status, fecundity, and age. Behavioral Ecology 22:184-191.

Martin, O. Y., and D. J. Hosken. 2002. Strategic ejaculation in the common dung fly Sepsis cynipsea. Animal Behaviour 63:541-546.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biological Reviews of the Cambridge Philosophical Society 45:525-&.

Pitnick, S. 1996. Investment in testes and the cost of making long sperm in Drosophila. American Naturalist 148:57-80.

Pitnick, S., R. Dobler, and D. J. Hosken. 2009. Sperm length is not influenced by haploid gene expression in the flies Drosophila melanogaster and Scathophaga stercoraria. Proceedings of the Royal Society B-Biological Sciences 276:4029-4034.

Puniamoorthy, N., M. A. Schäfer, and W. U. Blanckenhorn. 2012a. Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly, *Sepsis punctum* (Diptera: Sepsidae). Evolution 66:2117–2126.

Puniamoorthy, N., W. U. Blanckenhorn, and M. A. Schäfer. 2012b. Differential investment in pre- versus post-copulatory sexual selection reinforces a cross-continental reversal of sexual size dimorphism in *Sepsis punctum* (Diptera: Sepsidae). Journal of Evolutionary Biology 25:2253–2263.

Puslednik, L., R. C. Russell, and J. W. O. Ballard. 2012. Phylogeography of the medically important mosquito Aedes (Ochlerotatus) vigilax (Diptera: Culicidae) in Australasia. Journal of Biogeography 39:1333-1346.

Routto, J., D. Mazzi, K. Van Der Linde, P. Mirol, R. K. Butlin, and A. Hoikakala. 2007. The extent of variation in male song, wing and genital characters among allopatric Drosophila montana populations. Journal of Evolutionary Biology 20:1591-1601.
Schultz, K. S. 1999. The evolution of mating systems in black scavenger flies (Diptera: Sepsidae). University of Arizona, Tuscon, AZ.
Simmons, L. W., G. A. Parker, and P. Stockley. 1999. Sperm displacement in the yellow dung fly, Scatophaga stercoraria: An investigation of male and female processes. American Naturalist 153:302-314.
Symonds, M. R. E., A. Moussalli, and M. A. Elgar. 2009. The evolution of sex pheromones in an ecologically diverse genus of flies. Biological Journal of the Linnean Society 97:594-603.
Wojcieszek, J. M., and L. W. Simmons. 2012. Evidence for stabilizing selection and slow divergent evolution of male genitalia in a millipede (*Antichirpus variabilis*). Evolution 66:1138-1153.

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